

The evaluation of tests for the identification of semen

By

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List of abbreviations

Abbreviations	Definition
Abs	Absorbance units
ALS	alternate light source
AP	Acid phosphatase
DNA	deoxyribonucleic acid
FB	Fast Blue
FSL	Forensic Science Laboratory
KM	Kastle-Meyer
kDa	KiloDaltons
k_1	Enzyme-substrate association rate constant
k_2	Enzyme rate constant
k_{-1}	Enzyme-substrate disassociation rate constant
k_{cat}	Enzyme turn over number
microRNA	Micro RNA: RNA transcripts which are 2-8 nucleotides in length
mL	millilitre
mRNA	messenger RNA
nL	nanolitre
nm	nanometer
PSA	Prostate-specific antigen
PT	presumptive test
RNA	ribonucleic acid
s	seconds
SAPS	South African Police Service
Sg	Semenogelin
U	Enzyme unit: a unit is a measurement of the activity of the enzyme.
U/mL	Units per millilitre
UI	International unit: a unit denoting the biological activity
UV-vis	Ultraviolet-visible
μ L	microlitre
μ m	micrometer

Part A. Research Protocol

1 Summary

The stains found at the crime scene need to be screened to determine if the stain is of bodily origin (e.g. semen, blood and saliva etc.) and could be analysed for DNA. This is typically done by presumptive testing. Forensic scientists use chemical or enzymatic assays to determine the component of the stain to make an inference as to the identity of the stain. In the case of a possible semen stain, a test for acid phosphatase is used. Acid phosphatase (AP) is found at higher concentrations in semen than other bodily fluids. The AP test is a colour reaction that turns from colourless to purple in the presence of AP. At high levels of AP, this reaction can take a few seconds. Several other bodily fluids, as well as plant materials, can react with the AP test resulting in false positives. There is no consensus in the literature regarding the cut-off time for a positive reaction. South African Police Service (SAPS) Forensic Science Laboratory (FSL) use 50 seconds as the time for a positive human semen identification using the commercial brentamine Fast Blue (FB) reagent. In this study we aim to test the specificity of the AP test against non-human mammalian semen and well as plant extracts to determine the accuracy and reliability of the SAPS FSL time period for positive results. We will use both the direct and indirect method to apply the brentamine FB to controlled semen stains and will determine the reaction kinetics of this test.

2 Introduction

The detection and identification of stains as potential biological or bodily fluids at crime scenes, are vital steps in forensic investigations. The common bodily fluids present at most crime scenes are blood, vaginal fluid and semen [1]. These fluids could play a pivotal role in determining the sequence of events which occurred [1,2]. The presence of vaginal fluids and semen at a crime scene suggests a sexual encounter or assault had occurred, and blood suggests a physical altercation [1,3]. These bodily fluids are detected at the scene using a particular form of light commonly called an alternate light source (ALS) to detect and visualise the stain [2]. The bodily fluids from which the stain originate, would be identified using presumptive tests (chemical, catalytic, chemiluminescence or immunological tests). Confirmatory tests will then be carried out on the samples from the stains to verify the substance [2].

2.1 Detection and Presumptive testing

Since the naked eye on its own is sometimes insufficient to notice certain stains, the use of an ALS aids in this regard. The ALS emits light at a specific wavelength and certain fluids will fluoresce or darken when the ALS is shone upon the stain [4,5]. Semen contains 4-methylumbelliferyl phosphate, which is modified by acid phosphatase (AP), and the product fluoresces under ultra-violet light [6]. However, detection in this manner could give poor results when the stain is on dark or different types of fabrics [4,5]. Therefore, presumptive tests would be performed on suspected bodily stains to elucidate the source of the stain [1,2].

The presumptive tests for bodily fluids determine the identity of the fluid by testing for specific components. Blood, which contains haemoglobin, will fluoresce in the presence of Luminol, which reacts with iron [2]. Additionally, the Kastle-Meyer (KM) test presumptively determines the possible presence of blood. The KM test uses phenolphthalein and hydrogen peroxide to react with the haemoglobin in the stain resulting in a colour change [7]. To test for semen, a chemical/enzymatic, AP test and immunological prostate-specific antigen (PSA) test [8,9] can be used. Both these tests are able to detect semen in men who are azoospermic

(have a low sperm count) or have been vasectomised [10]. The AP test has been shown to be a good screening test to determine if there is semen on textiles [11].

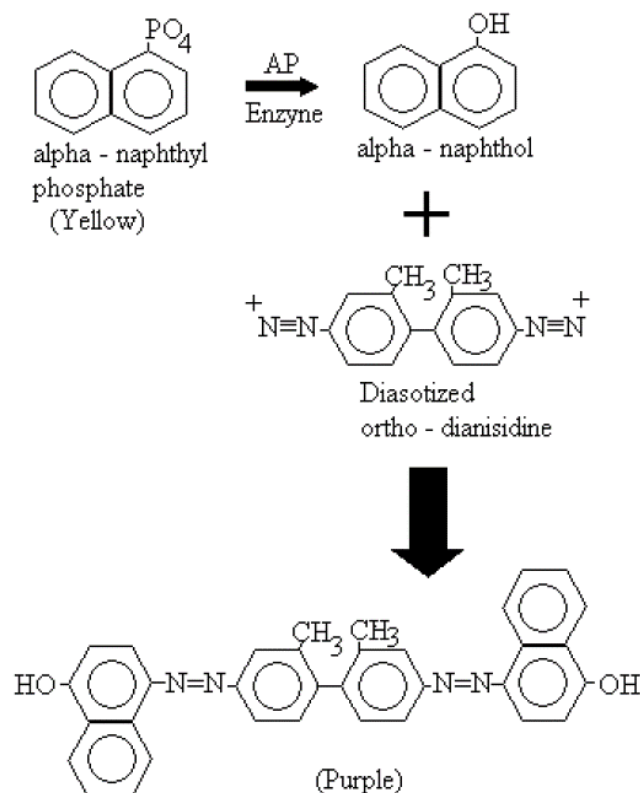


Figure 1 The reaction mechanism of the acid phosphatase presumptive test.

The AP test is a determination of the presence acid phosphatase enzyme in the stain. The enzyme occurs 500 to 1000 times greater in semen than to any other bodily fluid [1,8]. The test is based on the reaction shown in Figure 1, the AP removes the phosphate group of the substrate, α -naphthyl phosphate, which then becomes α -naphthol. The α -naphthol reacts with diazotised ortho-dianisidine (brentamine FB) which results in the formation of purple azo dye, over time [12,13]. There are several methods in which the AP test is performed: the indirect method, which is routinely used, when a moistened swab or blotting paper is applied to the stain and the swab or blotting paper is then tested for AP [12,14,15]. Conversely, the direct method directly treats the stain on the fabric with an aerosolized solution of the AP test compounds [3]. The direct method may be more sensitive at detecting AP in low concentrations of semen and did not appear to affect the subsequent DNA testing of semen stains [3]. Partial DNA profiles were amplified from 1 in 3000 dilution test directly whereas

for the indirect test a partial profile was amplified from the 1 in 2000 dilution [3]. Full profiles were amplified at the 1 in 750 dilution for the indirect test and the 1 in 500 dilution for the direct test [3].

The AP test is a presumptive test because other bodily fluids also contain acid phosphatase such as vaginal secretions and faeces. Presence of these fluids could result in a false positive identification of semen [3,16]. Certain plant materials have also been shown to give weak positive reactions [3,17]. The false positive results from non-seminal stains showcases the poor specificity of the AP test, as the false positives would give a faint colour change comparable to a dilute semen stain [3]. A study in the United Kingdom demonstrated that vaginal excretions and certain food products, for example, tea mushrooms and apple elicit a positive result when tested for the presence of AP [3]. Tea has been shown to react positively and relatively quickly when tested with the AP reagents, reacting within 30 seconds when indirectly tested and 7 seconds when directly tested [3]. The apple, grape and mushroom react with a weaker colour change that develops around one minute after treated with the AP reagents [3]. This non-specificity has lead forensic laboratories to put a time limit on the AP reaction. In the United Kingdom, the reaction cut-off is two minutes, but this cut-off could lead to the exclusion of dilute semen samples as true positive results [18,19]. The forensic community in South Africa uses 30 - 50 seconds as a cut-off time, assuming that if the stain has turned purple within 50 seconds, it has tested positive for human semen (Standard operating procedure of SAPS FSL).

2.2 *Confirmatory tests*

An important next step to determine the source of the bodily stain is use of a confirmatory test. For semen stains, this is sperm cytology. Cells are extracted from the stain using buffers, the resulting extracted solution, presumably containing sperm, is then mounted on a microscopic slide and stained with either haematoxylin and eosin or the Christmas tree stain [20–22]. The slides are then scored to determine the number of sperm cells found and if they contain tails. This can be used to determine the approximate time since intercourse and draw time inferences for the alleged incident [23]. However, Tobe *et al.* demonstrated a lack of consensus on the scores of 37 forensic scientists scoring the same slides [24], suggesting that

this test is highly subjective. The Christmas tree stain can also be used to distinguish human sperm cells from those of other mammals [25].

Genetic profiling is used as a confirmatory test for semen stains [20,26–28]. DNA profiling is performed on samples where the presence of sperm cells are confirmed but has also been shown to identify male-specific DNA from stains which are absent of sperm [10,11]. The DNA analysis targets the Y-chromosome, which is male-specific. The AP test is unlikely to interfere with the amplification process of the subsequent DNA profiling of semen stains [29].

2.3 Enzyme kinetics

For presumptive or confirmatory test that use enzymatic reactions the rate of the particular enzyme could be used as a discriminator. The primary function of enzymes is to enhance the rates of reactions [30]. The rate of catalysis or velocity of the enzyme is defined as the number of moles of the product formed per second, which is directly proportional to the concentration of the reactants i.e. the enzyme and substrate [30,31]. The Michaelis-Menten model (Figure 2) states an enzyme E binds to the substrate A to form the enzyme-substrate complex EA at a rate constant of k_1 . The EA complex has two possible outcomes, either to dissociate to E and S at a rate constant k_{-1} or it can proceed to form the product P with a rate constant of k_2 [32]. This reaction assumes that the product does not revert to the initial substrate, therefore making the reaction irreversible [30].

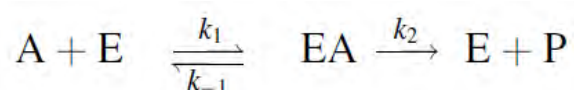


Figure 2 The Michaelis-Menten equation for an irreversible enzymatic reaction.

If the reaction of substrate to product is a colour reaction the amount of colour change can be measured using a spectrophotometer. A colour change means that a specific wavelength of light is being absorbed by the product [31]. The technique, absorption spectroscopy uses the

attenuation of light intensity at a particle wavelength after passing through a solution of an absorbing compound to measure the concentration of the particular compound [31].

3 Rationale

The acid phosphatase (AP) presumptive test used in the South African Police Services (SAPS) needs to be evaluated such that it is commensurate with international standards. There is no consensus with regards to the cut-off time to determine a positive semen stain. Ideally a validated cut-off time is required to exclude stains that could potentially be false positives and decrease the number of false negatives. The cut-off time should not be biased against diluted stains. To date, the validation of the cut-off time has been studied qualitatively, in this study we aim to determine the cut-off quantitatively using reaction kinetics of the AP enzyme.

4 Aims and objectives

4.1 Aims

The aim of the study is to investigate the AP test using brentamine FB presumptive test for semen with regards to its kinetics, sensitivity, and specificity to humans.

4.2 Objectives

To fulfil these aims, the objectives are to:

- Compare the direct and indirect method of brentamine FB testing on semen stains.
- Establish the reaction time of the brentamine FB colour change test on human and non-human mammal semen samples (comprising a range of ages and concentrations) as well as substances that have been reported to cause false positives or negatives; both visually and by objective kinetics measurement.
- Assess the accuracy and reliability of using a reaction time of 5 seconds to indicate a positive human semen sample

5 Proposed research methodology

5.1 Study paradigm

This is a cross sectional study with a target group being human and non-human males. This a quantitative study measuring the association of a positive acid phosphatase test reaction (dependent variable) and the activity of the acid phosphatase enzyme (independent) in seminal fluid.

5.2 Study Populations

5.2.1 Humans

The cohort will consist of approximately 30 male adult volunteers older than 18 years of age. The volunteers will be approached personally by the forensic scientist from within the University of Cape Town and outside. A power calculation could not be used to verify the population size, as no prevalence or incidence variable is available.

Inclusion criteria:

- Must be male
- Consented to be a volunteer (refer to appendices of informed consent)
- To their knowledge be free of all sexually transmitted infections and diseases of the genital tract

5.2.2 Non-human males

Semen will be collected from five pigs, five bulls and five rams. The non-human mammals are held at the Elsenburg farm. The semen samples will be obtained through their veterinary service division. The semen samples will be obtained using electro-ejaculation, which is a method that is approved by the farm's ethics board.

Exclusion: Any non-human mammal semen acquired from means which did not meet the ethical requirements.

6 Laboratory work

The volumes used in this study were chosen arbitrarily as starting point for this study, as there is no standard existing protocol and a study of this nature has not been carried out in South Africa before.

6.1 Collection of samples

Volunteers will be asked to collect early morning ejaculate in the specimen tube provided the day before. The volunteers will be required to masturbate and ejaculate into the sample tube and record the time at which this was done. The volunteers will return the specimen tube with ejaculate as soon as possible after collection. The volunteers will be required to donate twice during the duration of the study. A second date for donation will be determined by the forensic scientist and the volunteer.

6.2 Sensitivity of AP test

A number of dilutions (1 in 4, 1 in 10, 1 in 40, 1 in 100, 1 in 400, 1 in 1000, 1 in 2000 and 1 in 4000) will be made for each fresh semen sample using distilled water. 200 µL of the dilutions and neat semen will be seeded onto sections (5 cm x 5 cm) of white poly-cotton fabric in duplicate. Each stained cotton section will be allowed to dry at room temperature for one hour then be tested with the AP test reagents directly and indirectly as per Lewis *et al* 2013 and Davidson *et al* 2012 using a spray bottle [3,15].

In addition, another duplicate set of stained poly-cotton fabric will be made, as described above, and allowed to dry for one week at room temperature. These aged stains will then be treated with AP test reagents directly and indirectly. The time in which the stained cotton sections and blotting paper turn purple will be recorded.

6.3 Specificity of the AP test

To test the specificity of AP test, dilutions of non-human mammal (pig, ram and bull) semen will also be made and seeded onto poly-cotton material pieces and tested directly and indirectly using a spray bottle containing the AP test reagents. Extracts from uncooked

mushroom, grape (white and red) and apple, as well as tea (Ceylon and Rooibos) will be brewed. All these extracts have been shown to react with the AP test reagents with varying degrees [3]. The extracts will be made by grinding the material in a mortar and pestle to create a paste and seeded onto poly-cotton sections. The teas will be brewed in 200 mL of boiling water and 15 μ L of the brew will be seeded onto the poly cotton fabric [17]. The stained fabric will be treated directly and indirectly with the AP test reagents. The time in which the stained cotton sections and blotting paper turn purple will be recorded. To test for inhibition fresh neat semen stains will be sprayed with a 1% detergent solution before being treated with the AP test reagents.

6.4 Reaction kinetics of the AP test

The dilutions of fresh and one week aged human and non-human mammal semen will be aliquoted into plastic spectrophotometric cuvettes, the AP test reagents will be added to the cuvettes. A number of dilutions of AP enzyme will be aliquoted into cuvettes. The cuvettes will be placed in a UV-VIS spectrophotometer and set to measure the absorbance at 525 nm [13]. For each dilution the absorbance values will be recorded and plotted. Using Michaelis-Menten enzyme kinetics equations, the reaction kinetics will be determined for AP enzyme and the human and non-human mammal samples.

6.5 Sperm cytology

The human and non-human mammal semen samples will be mounted onto microscopic slide treated with Histobond®. The slides will be stained with haematoxylin and eosin and viewed under a compound microscope. The slides will be compared to determine if there are apparent morphological differences between human, pig, ram and bull sperm cells.

6.6 Equipment and reagents

For this study the use of a UV-Vis spectrophotometer and light microscope are required. The AP test reagent will be made up as follows:

Stock solution A

Brentamine FB

1g

Sodium acetate	20g
Acetic acid	10ml
Water	100ml

Stock solution B

Sodium α -naphthyl phosphate	0.8g
Water	10ml

The working AP test reagents is prepared by adding 10ml of stock solution A to 89ml of water and then adding 1ml of stock solution B [17]. The test AP reagents are then keep in a 4 °C fridge until use.

Haematoxylin and eosin are required for the staining of the sperm cells.

6.7 *Analysis of results*

The Student t-test will be used to determine if there is significant difference between the kinetic of human semen versus the false positives and the non-human mammal semen. The null hypothesis of the test will be there is no significant difference between the enzyme kinetics, and the alternate hypothesis will state there is a significant difference between the enzyme kinetics.

7 Outcomes and social value

The outcome of the study will inform forensic procedures of screening possible seminal stains at crime scenes and in the forensic laboratory. If the enzyme kinetics of human semen and non-human mammal semen are no different, the inference that a positive AP reaction within 50 seconds indicating a human sample is unreliable and should be altered in the SAPS FSL standard operating procedures accordingly. The study would aid in determining an improved time threshold. This study could also aid the identification of non-human mammal semen at the crime scene. The recognition of non-human mammal sperm cells in the stain would aid in better determination of the source of the stain.

8 Ethics and risk assessment

This study is subject to the approval of the Research Ethics Committee and if approved, it will be executed in accordance with the declaration of Helsinki (1964) as amended, most recently at the fourth General Assembly in Fortaleza, Brazil, October 2013. This study will also be subject to approval of the Human and Animal Ethics Committees of the University of Cape Town.

8.1 Adverse effects to participants

There will be no adverse effects to the participants.

8.2 Risk to the researcher

There is a risk of semen to skin exposure to infection diseases such as common sexually transmitted diseases and genital tract infections. The consequence of these risks is moderate to major as certain infections are worse than others and the infections are treatable. The likelihood of the risk occurring is rare, making the risk assessment low. To mitigate the occurrence of possible exposure to infectious samples personal protective equipment will be worn, this includes latex gloves, a lab coat and safety glasses.

8.3 Confidentiality

The semen samples will be labelled with an alphanumeric code which will correspond to a signed consent form which will also be labelled with a code. The forms will be kept in the office of supervisor under lock and key.

9 Research Protocol Appendices:

INFORMATION FORM and INFORMED CONSENT

Project Title: **The evaluation of tests for the identification of semen**

Researcher: Lyle Curry

Supervisor: Laura Heathfield

We would like to invite you to participate in a research study that involves forensic presumptive testing for semen, using the acid phosphatase test. The recruitment of this study will end on the 15 November 2015.

The decision to participate is entirely your own. If you decide not to participate in the study this will not disadvantage you in any way. There is no obligation on you to participate in this study. In addition, at any point during the study you are free to withdraw without having to provide any reason for this, and with no consequences to you. There is no monetary incentive for this study.

Background:

Presumptive testing of stains at the crime scene or in the forensic lab are quick methods of possible identification of the body fluid responsible for the stain. Forensic scientists use the acid phosphatase test for the presumptive identification of semen. Acid phosphatase (AP) is an enzyme that is found in high concentrations in semen. The test for AP involves treating a stain with a mixture of reagents and if the stain is positive for AP the stain will turn purple. In the Forensic Science Laboratory in South Africa, the cut-off time for a positive reaction for the AP test is 50 seconds, which in the eyes of the forensic analysts, indicates the possible presence of human semen. This cut-off time however, has not been tested in the lab, nor has the AP test been compared to semen samples from animals. The aim of this study is to therefore evaluate exactly how long the AP test takes to presumptively identify semen and to investigate how specific this test is to humans. We will also test the sensitivity of the AP test by aging semen stains for one week and comparing the time to a positive reaction against a fresh semen stain.

The study will involve collecting semen from male volunteers and various animals. The donated semen samples will be tested to investigate how specific the AP test is on human semen when compared to animal semen and to determine the reaction kinetics of the AP enzyme in semen stains. This will allow us to better determine the cut-off time for the AP test in order to better inform forensic labs on what the cut-off time should actually be. No DNA analysis will be carried out on the semen samples.

What we need from you:

To participate, you will need to donate two semen samples. You will be required to masturbate and ejaculate into the sample jar and record the time at which this was done. This can be done in the comfort of your own home. There will be no risk your health.

The donation must be done in the morning to ensure a fresh sample to be used in the study. The sealed specimen jar with ejaculate must be returned to the researcher as soon as possible after collection. If there is any time delay between sample collection and handing it to the researcher, the sample must be kept in the fridge. The second semen donation must take place one week after the first donation. This will be used to create a paired comparison to the one week-aged stain.

You will be require to remain abstinent for 36 hours before making a donation, that is no ejaculations 36 hours before the donation. You will also be required to keep the semen from making any contact with any other bodily fluids. The samples will be anonymised and coded, and stored in a 4° C fridge within an access controlled laboratory in the Division of Forensic Medicine and Toxicology until the project is complete. If you should decide to withdraw from the study at any time, your samples will be destroyed.

All information about you will be kept strictly private and confidential. You will not be given the results of any tests done.

Contact details:

This study has gained ethics approval from the Faculty of Health Science Human Research Ethics Committee (HREC 502/2015). The UCT's Faculty of Health Sciences Human Research Ethics Committee can be contacted on 021 406 6338 in case participants have any questions regarding their rights and welfare as research subjects on this study. You may also contact the study supervisor Ms Laura Heathfield at telephone 021 406 6569 or email: laura.heathfield@uct.ac.za.

Please read the sentences on the accompanying consent form and then tick either the yes or no box.

Thank you for your participation and your valuable time.

INFORMED CONSENT

I, _____ (full name),
do willing state:

	Yes	No
I have read the above / the above has been read to me, and I understand what this study entails.		
I have had the opportunity to discuss the study and my questions have been answered to my satisfaction.		
I know what is required of me, and I understand and accept the requirements.		
I consent to participate in this study I understand that my consent is entirely voluntary.		
I understand that I may withdraw from the research study at any time without giving a reason and without any consequence to me whatsoever.		
I understand that if I withdraw from participation at any time, my samples will be destroyed.		

I understand the samples will be stored for the duration of the project in the Division of Forensic Medicine and Toxicology at the University of Cape Town. After the project, I would like (please tick one option):

- ☐ The samples to be stored for future research for any research project which is approved by the Faculty Health Science Human Research Ethics Committee.
- ☐ The samples to be stored for future research that stems directly from this research project and which is approved by the Faculty Health Science Human Research Ethics Committee.
- ☐ The samples to be destroyed.

If you sign this form, it means that you voluntarily give permission to participate in the study.

Participant:

Print Name:

Signature:

Date: |_|_|/|_|_|/|_|_|_|_|

Person obtaining consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer:

Print Name:

Signature:

Date: |_|_|/|_|_|/|_|_|_|_|

Witness

Print Name:

Signature:

Date: |_|_|/|_|_|/|_|_|_|_|

PARTICIPANT QUESTIONNAIRE:

Date of birth:

YES NO

To the best of your knowledge, do you have any sexually transmitted infection or diseases?		
Have you had a vasectomy?		
Are you willing to donate semen more than once?		

Date & time of first donation: |_|_|/|_|_|/|_|_|_|_| Time:

Date & time of second donation: |_|_|/|_|_|/|_|_|_|_| Time:

Amendments to Protocol

5.2 Study Populations

Humans

The cohort will consist of approximately 30 male adult volunteers older than 18 years of age. The volunteers will be approached personally by the forensic scientist from within the University of Cape Town and outside.

This was changed to 15 male adult participants due to time constraints.

Non-human males

Semen will be collected from five pigs, five bulls and five rams.

This was amended to two male canines. The contact for the livestock samples did not return any correspondence after initially agreeing to supply specimens.

Sensitivity of AP test

Each stained cotton section will be allowed to dry at room temperature for one hour then either be tested with the AP test reagents directly and indirectly as per Lewis *et al* 2013 and Davidson *et al* 2012 using a spray bottle.

Direct application of 50 µl of the brentamine FB reagent was used instead. This was done in order to keep the ratio of semen volume and reagent constant between the enzyme kinetic experiments.

Amendment to aging of stains

Originally, another duplicate set of stained poly-cotton fabric will be made, as described above, and allowed to dry for one week at room temperature.

The aging period was increased to two weeks was used. During the initial stages of the kinetic experiments a technical difficulty arose with the apparatus initially required for the experiments but the first participant had done the first donation one week prior. Therefore to

change apparatus and allow for the participant's the second donation to be used in the study the aging time was increased.

Specificity of the AP test

To test for inhibition fresh neat semen stains will be sprayed with a 1% detergent solution before being treated with the AP test reagents.

Due to time constraints the experiment above was left out of the study.

Sperm cytology

The slides will be compared to determine if there are apparent morphological differences between human, pig, ram and bull sperm cells.

As previously stated, canine semen was used.

Equipment and reagents

The AP test reagent will be made up as follows:

Stock solution A

Brentamine FB	1 g (0.5 g)
Sodium acetate	20 g (10 g)
Acetic acid	10 mL (5 mL)
Water	100 mL (50 mL)

Stock solution B

Sodium α -naphthyl phosphate	0.8 g (0.4 g)
Water	10 mL (5mL)

The working AP test reagents is prepared by adding 10ml (5mL) of stock solution A to 89ml (44.5 mL) of water and then adding 1mL (0.5 mL) of stock solution B.

The quantities were halved (amended quantity in brackets), this was because the reagent needed to be made fresh every day and the reduction in quantities, reduced wastage.

5.3.3 Analysis of results

The **Student t-test** will be used to determine if there is significant difference between the **kinetic of human semen versus the false positives and the non-human mammal semen**. The null hypothesis of the test will be there is no significant difference between the enzyme kinetics, and the alternate hypothesis will state there is a significant difference between the enzyme kinetics.

A **Wilcoxon matched test** was performed, this was because the samples being tested were non parametric; the sample size was too small ($n = 15$) and the data was exponential not normally distributed. The test was also used on all of the **human specimens: direct test and indirect: aged vs fresh, as well as the reaction rates: fresh and aged**. The test could not be performed on the canine sample as there were only 2 participants.

Part B. Literature Review

Background

The detection and identification of bodily fluids at crime scenes and/or in the lab are vital steps in forensic investigations. The common bodily fluids present at crime scenes are blood, semen, saliva and vaginal fluid [1]. These fluids could play a pivotal role in determining the events which occurred and possibly, the sequence thereof [1,2]. The presence of vaginal fluids and semen at a crime scene suggests a sexual encounter or assault had occurred, and blood suggests a physical altercation [1,3]. Further, the identification of a possible bodily fluid typically prompts the collection of that sample for downstream DNA analysis, for identification purposes.

These bodily fluids are detected at the scene using a particular form of light commonly called an alternate light source (ALS) to detect and visualise the stain [2]. The possible type of bodily stain would be screened using presumptive tests (e.g. chemical colour change tests, chemiluminescence or immunological tests). Confirmatory tests will then be carried out on the samples from the stains to verify the substance [2].

Semen

Semen is an organic fluid which is produced by the male species. Semen is made up of spermatozoa (sperm cells) and seminal fluid which is secreted from different glands. The spermatozoa are produced in the testes and the fully matured spermatozoa then flow up the vas deferens to combine with the secretions of the seminal vesicles and the prostate, followed by the periurethral glands to form semen [33,34]. The typical volume of ejaculate is at least 2 mL with a pH of 7-8 and a spermatozoa concentration of 20-150 million cells per mL [35–38].

Spermatozoa

Sperm cells are produced in the testes and flow into the epididymis where the sperm cells are stored and undergo maturation before mixing with seminal fluid [39]. The sperm cell comprises three distinct parts: the head, the neck and the tail (Figure 1) .

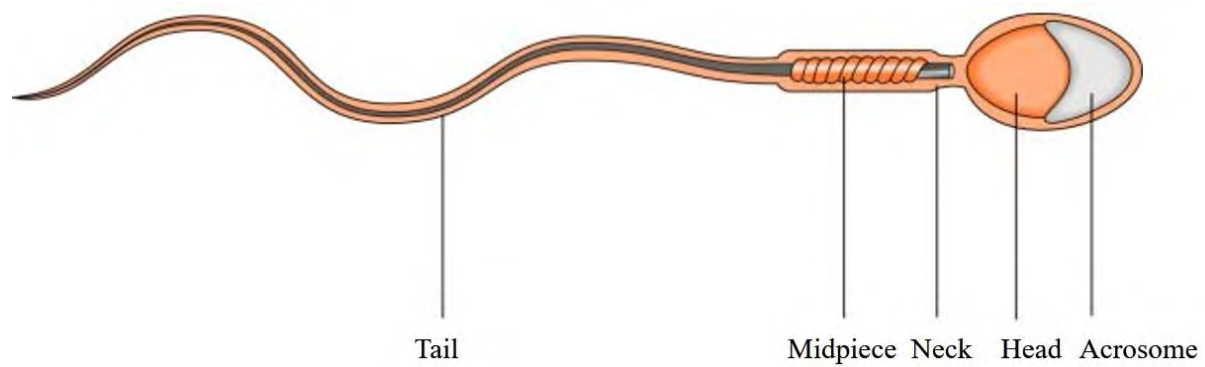


Figure 1 A schematic diagram of a mammalian spermatozoa. The main features of the spermatozoa are labelled: the tail, midpiece neck, and head. The acrosome is the vesicle that contains digestive enzymes that breakdown the outer layer of the ovum

The lengths of these parts vary between and within species [40]. The length of the spermatozoa of different mammalian species and the dimensions of the parts of the cell are shown in table 1 [40]; the length of the human sperm cell is relatively shorter than of the horse, cattle, and sheep, whereas the human cell is comparable to the pig cell. The head of the human sperm cell is smaller in length and width when compared to the other mammals. The neck of the human sperm cells is almost three times smaller than those of the other mammals. The tail of the human cell is one of the longest but is comparable to the sheep and cattle sperm cells.

Table 1: Dimensions of different mammalian sperm cells (μm) [40]

Species	Total Length	Head		Neck		Tail	
		Length	Width	Length	Width	Length	Width
Man	50-60	3.4-4.6	1.5-2.8	3.5-5.0	0.8-0.6	44-50	0.5-0.4
Stallion	60	5	2.4	8	0.5	30	0.49
Bull	75-90	9.15	4.25	14.84	0.67	50	0.51
Boar	50-60	8.5	4.25	10	-	30	-
Ram (sheep)	70-80	8.2	4.25	14	0.8	45	0.5

- Indicates no measurement

The sperm head contains the acrosome, a vesicle that contains a hydrolytic enzyme which breaks down the egg wall, and the nucleus which contains the DNA. The head shape and size shows high variation and is species-specific (Figure 2). The human sperm has a flat disc-shaped head with a slightly pointed tip (Figure 2F); the guinea pig (Figure 2A), rabbit (Figure 2B) and dog sperm (Figure 2E) have a similar shape but the heads are relatively smaller and have a rounded tip. The mouse sperm cell head has a hook shape (Figure. 2C). The horse sperm cell head is similar in size (table 1) to the human sperm cell but the shape is dissimilar (Figure 2D). The neck of the sperm cell connects the tail to the head and contains the mitochondria that is the power supply to move the tail for locomotion [40].

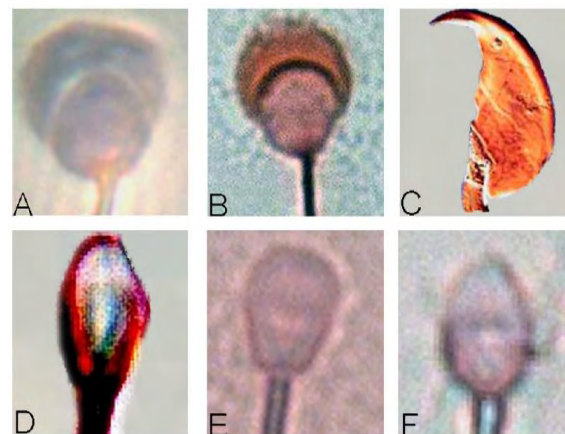


Figure 2: Sperm heads of different mammals in light microscopy and stained with eosin. A) guinea pig, B) rabbit, C) mouse, D) horse, E) dog and F) human [40]

Components of seminal fluid

Seminal fluid is made up of carbohydrates, proteins, peptides and lipids in an aqueous medium [33,34]. The constituents of seminal fluid are secreted from different sources which include: epididymis, the vas deferens, seminal vesicles, prostate gland and periurethral glands [33]. Seminal fluid contains high concentrations of glucose and inorganic phosphorus [41] and that from fertile human individuals contain over 200-300 proteins and protein subunits, with a range of sizes [42]. A study by Edwards *et al.* (1981) identified the differences in composition of seminal fluid between fertile and vasectomised males. They showed that certain proteins (with the approximate mass of 30 kDa) were absent in vasectomised males.

These absent proteins were most likely of testicular origin. Acid phosphatase (AP) however, was present in both vasectomised and fertile males suggesting the enzyme originates in the prostate [42]. A study by Autiero *et al* 1991, identified a relative change in ratios of seminal proteins of infertile (oligospermic: low sperm count and asthenospermic: immotile sperm) men [43]. They showed AP was still present and functioning in the semen of infertile men [43].

Prostate-specific antigen (PSA) was originally characterised as a semen specific substance that could be used as forensic evidence that would aid in rape cases [44]. PSA was identified and purified by different research groups and given different names over time from 1973 to 1979 [45–47] and this has created controversy as to who is attributed to initially discovering PSA [48]. Many modified forms of PSA were also identified in seminal fluid [33]. PSA is a glycoprotein produced by the prostate gland and is found in the seminal fluid at concentrations ranging from 0.5-3.0 mg/mL [49]. The main function of PSA is the liquefaction of seminal fluid [49–51]. Both AP and PSA have also been shown to be biomarkers for the diagnosis of prostate cancer [52,53].

Semenogelin (Sg) is a protein which is secreted by the seminal vesicles [54]. There are two variants of the protein called Semenogelin 1 (Sg1) and Semenogelin 2 (Sg2), these proteins make up 20-40% of the seminal fluid proteins [55,56]. Sg1 and Sg2, after ejaculation, form a gel-matrix which immobilises the spermatozoa and prevents premature capacitation, i.e. the maturation of the spermatozoa to be ready for fertilisation [57,58]. After 15-20 minutes have passed, post-ejaculation, the gel matrix is liquefied by the degradation of the semenogelins by PSA [50,51].

The AP enzyme occurs 500 to 1000 times greater in semen than to any other bodily fluid [1,8]. Human prostatic AP is a 100 kDa glycoprotein composed of two subunits, and is expressed in well-differentiated normal human prostate epithelial cells [59,60]. AP enzymes hydrolyse a wide assortment of small organic phosphomonoesters under acid conditions where optimum pH is 4-6.2. The physiological function has not been uncovered yet but it may be associated with the liquefaction of semen [60–63].

Detection of bodily fluids

Since the naked eye on its own is sometimes insufficient to notice certain stains, the use of an ALS aids in this regard. The ALS emits light at a specific wavelength and certain fluids will fluoresce or darken when the ALS is shone upon the stain. Forensic scientists can also wear coloured goggles to increase the contrast of the fluorescing stain by filtering out a specific wavelength of light [4,5]. For the detection of semen, the Wood's lamp (WL) is used. The WL emits a wavelength of light at 360 nm but semen does not fluoresce under this light [4]. Semen contains 4-methylumbelliferyl phosphate, which is modified by acid phosphatase (AP), and the product fluoresces under ultra-violet light [6]. Using WL to detect semen has a low specificity and thus the risk of detecting false positive is likely [4]. However, the purpose of an ALS is to help detect the presence of a possible bodily stain, and not to determine its identity.

The Polilight® PL500 is a multifunctional ALS and it can be used to detect saliva, blood and semen [64]. The PL500 has settings and filters that change the wavelength of the light emitted; it can emit light at 415-650 nm as well as white light and ultraviolet [64]. However, detection of semen in this manner could give poor results when the stain is on dark or different types of fabrics [4,5].

Once a possible bodily fluid has been found, presumptive tests would be performed on the stain to elucidate the possible type of stain, as ALS does not inform the forensic scientist of the contents of the stain [1,2].

Presumptive tests

Presumptive tests are efficient, fairly easy to use and are inexpensive tests to perform on stains at the crime scene or in the laboratory [65–67]. A presumptive test is a method to test for bodily fluids to determine the possible identity of the fluid by testing for class components that are relatively specific to that fluid. Presumptive tests are not considered specific enough to indicate unequivocally that a particular stain is a bodily fluid. This disadvantage can lead to other fluids, that may contain the class compound tested for, yielding a false positive result. Vaginal fluid and saliva, as well as certain food products, have

been shown to give a positive reaction when tested for AP using the AP presumptive test [3]. This is why confirmatory tests are required to further establish the identity of the stain [65].

Presumptive testing of semen

To test for semen, a chemical enzymatic test (acid phosphatase test) or an immunological test (PSA and semenogelin test) [8,9] can be used. These tests specifically target the seminal fluid component of semen. Therefore these tests are able to detect seminal fluid in men who are azoospermic or oligospermic, (have a low sperm count) or have been vasectomised [10,68]. The absence of spermatozoa from semen does not hinder the effectiveness of these presumptive tests, as AP and PSA are produced in the prostate and Sg is produced in the seminal vesicles, which are not effected by vasectomies.

PSA test

A presumptive test for semen is the PSA test which is carried out using an enzyme-linked immunosorbent assay (ELISA) which uses antibodies specific to PSA [9,28,49,69]. These antibodies are situated on a membrane housed in a plastic cassette. The mechanism of the test is shown in figure 2. The sample containing PSA (antigen) is added through the application port of the cassette which contains the mobile antihuman PSA antibody dye conjugate which combines to form the mobile antigen-antibody complex. The mobile complex then migrates down the membrane where it is captured by the immobile antihuman PSA antibody. As more of the complex is captured by the immobile antibody, the amount of dye increases, which leads to a blue stripe being seen on the membrane. As an internal control, unbound mobile antihuman PSA antibody is captured by an immobile anti-Ig-antibody (an antibody that binds to antibodies) and this forms another blue stripe further down the membrane [69].

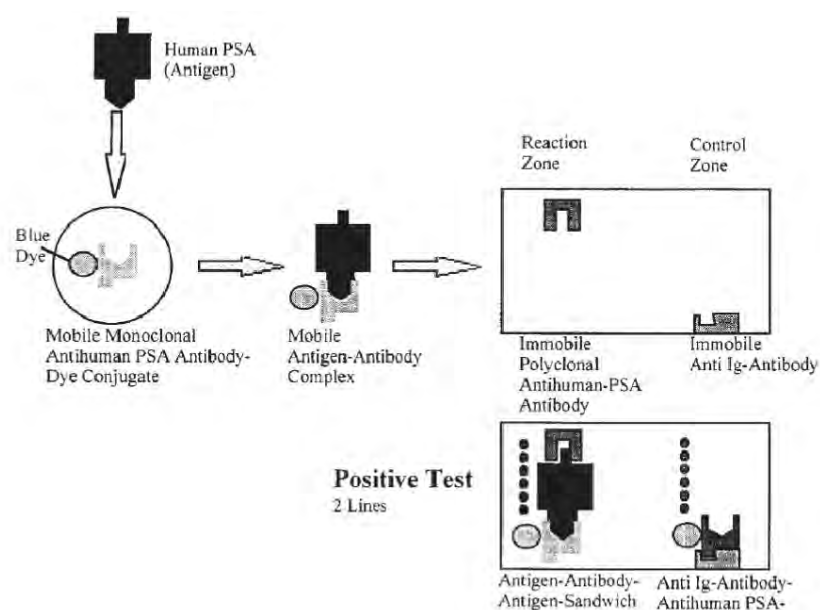


Figure 3: A schematic diagram illustrating the mechanism of the PSA membrane test [69]. The circle on the left indicates the sample application port of the cassette. The rectangle on the right indicates the viewing port of the cassette

PSA can be found at lower levels in other bodily fluids, most notably female breast milk and female urine, but these levels are generally below detection for forensic presumptive test kits [28]. This means that the possibility of getting a false positive for PSA from other bodily fluids is low. However, there are certain condom brands that have shown to react and produce false positive reactions for the PSA presumptive test, especially if the condoms contained spermicide [70]. At high concentrations of PSA (greater than 50 mg/mL) certain PSA presumptive test kits do not give a positive result; this is called the high-dose Hook effect [69,71]. The Hook effect, in this instance, would mean that high concentrations of PSA could result in a false negative for that stain or sample when using the PSA testing kit. This could mean the exclusion of that stain or sample as evidence, as it did not react even though it contained PSA. The manufacturer's advice to counter this effect is to dilute the possible seminal fluid before testing [69]. Diluting the sample could reduce the concentration of the PSA to levels below where the hook effect would take place, thereby reducing the risk for a false negative reaction.

AP test

The presumptive test for AP has been shown to be an effective screening test to determine the presence of semen [11]. The test is based on the reaction shown in figure 3; the AP removes

the phosphate group of the substrate, α -naphthyl phosphate, which then becomes α -naphthol. The α -naphthol reacts with diazotised ortho-dianisidine (brentamine FB) which results in the formation of purple azo dye, over time [12,13].

There are several methods in which the AP test is performed: the indirect method, which is routinely used, is when a moistened swab or filter paper is applied to the stain and the swab or filter paper is then tested for AP [12,14,15]. Conversely, the direct method directly treats the stain on the fabric with an aerosolised solution of the AP test compounds [3]. The direct method has shown to be more sensitive at detecting AP in low concentrations of semen, as direct application of the AP reagent does not involve the dilution or the transfer of the stain. The direct method does not seem to affect the subsequent DNA testing of semen stains but this method can only be used once per stain [3]. The presence of the brentamine FB reagent did not affect the uptake of haematoxylin and eosin staining, during the confirmation analysis of spermatozoa [14]. The direct method could be problematic when the stain occurs on dark fabric, as the shade of the fabric could mask the possible colour change.

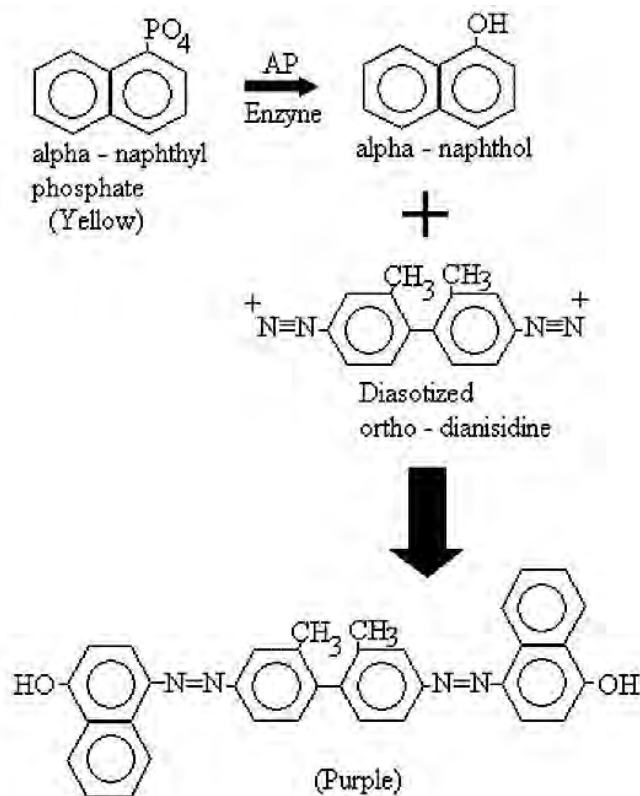


Figure 4: The reaction mechanism of the acid phosphatase presumptive test.

Other bodily fluids also contain AP such as vaginal secretions and faeces. Presence of these fluids could result in a false positive identification of semen [3,16]. Certain plant materials have also been shown to give weak positive reactions [3,16,17]. The false positive results from non-seminal stains showcases the poor specificity of the AP test, as the false positives would give a faint colour change comparable to a dilute semen stain [3,72]. This non-specificity has led forensic laboratories to put a time limit on the AP reaction. In the United Kingdom and Ireland, the observation of the reaction is recorded after two minutes [3,14,18,19], but this cut-off could lead to the exclusion of dilute semen samples as true positive results. Direct application of the AP reagents to dilute samples have been shown to react within one minute [73]. False positive reactions could also arise as non-semen stains could turn positive within a certain amount of time has passed [19].

The Royal Canadian Mounted Police used a cut-off time of 90 seconds, where if a stain turns an intense purple within the time it is considered a presumptive positive for semen [74]. The South African Police Service Forensic Science Laboratory (SAPS FSL) uses 65 seconds as a cut-off time, and if the stain has turned purple within 50 seconds, it is considered positive for human semen (Standard Operating Procedure (SOP) of SAPS FSL). Despite the SOP currently in use by SAPS FSL, there is no verification, confirmation or research supporting this particular cut-off time. The SAPS cut-off time is less than those used in the UK and Canada, and using this lower cut-off time may exclude true positives that are older or dilute that may not have reacted within the 65 second time period. The UK uses a cut-off time of two minutes but has been challenged as researchers have shown that dilute semen could give a positive reaction within 10 minutes [18]. Researchers could not find any literature for the basis of this 2 minute cut-off time that was employed in the UK [14]. However, increasing the cut-off could also increase the potential for the detection of false positives [3]. Therefore it is important to have an optimal cut-off time [18,19].

Enzyme kinetics

The primary function of enzymes is to enhance the rates of reactions [30]. The rate of catalysis or velocity of the enzyme is defined as the number of moles of the product formed

per second, which is directly proportional to the concentration of the reactants i.e. the enzyme and substrate [30,31]. The rate of a given reaction is used to determine the specific reaction order for that reaction. The order of the reaction is based on the reactants, particularly the amount of molecules of the substrate required to produce one molecule of product [31].

Using figure 4 as an example, the reaction catalysed by AP: α -naphthyl phosphate to α -naphthol, is a first-order reaction as one molecule of α -naphthyl phosphate is converted into α -naphthol. The colour reaction of α -naphthol and brentamine FB to the purple azo dye is a second-order reaction in terms of α -naphthol as two molecules of α -naphthol are required to make one dye molecule. The reaction is a first order reaction in terms of brentamine FB as only one brentamine FB molecule is needed.

The Michaelis-Menten model (Figure 4) states an enzyme E binds to the substrate A to form the enzyme-substrate complex EA at a rate constant of k_1 . The EA complex has two possible outcomes, either to dissociate to E and S at a rate constant k_{-1} or it can proceed to form the product P with a rate constant of k_2 [32]. This reaction assumes that the product does not revert to the initial substrate, therefore making the reaction irreversible [30]. The k_2 rate constant is also refer to as k_{cat} or turn over number of the enzyme, this is defined as “the number of substrate molecules converted into product molecules by an enzyme unit in a given unit of time, when the enzyme is fully saturated” [30].



Figure 5: The Michaelis-Menten equation for an irreversible enzymatic reaction.

The kinetics of human prostatic AP was studied by Schönheyder, but his work was carried out on unpurified AP. The reaction he was studying used AP to hydrolyse phenyl phosphate into phenol, and claimed that this reaction did not fit in the zero or first order kinetics. He postulated that, if purified enzyme was used, a better understanding of the kinetics of AP would be uncovered [63]. The catalysis of *p*-nitrophenyl phosphate by AP, was shown

however, to deviate from Michaelis-Menten kinetics [75]. The k_{cat} or turn over number of human prostatic acid phosphatase was determined to be greater than 500 per second (i.e. > 500 molecules of α -naphthyl phosphate was converted to α -naphthol per second) [76]. This study also determined the hill coefficient of AP; the Hill co-efficient is the measurement of the binding of the substrate to the enzyme. They determined the hill coefficient increased with the increase of AP concentration [76].

Studies into the activity of human AP have shown a varied range of enzyme activities [77–80]. The studies showed a biological variation with the human population with a range of mean AP activity per volume of semen as follows: 220 U/mL [77], 250 – 1300 U/mL, 480 U/mL [78] and an enzyme concentration of 1031 U/L in Miteva *et al.* (2010). Studies were performed on the enzymatic activity of non-human mammalian AP, showed the mean enzyme concentration as follows: 24.7 UI for bulls [81], 64.9 UI for rams [82], 680 UI for pig [83] and 11.5 for donkeys [80].

If the reaction of substrate to product results in a colour change of the solution, the amount of colour change can be measured using an ultraviolet-visible (UV-Vis) spectrophotometer. The colour of the solution is due the absorption of a specific wavelength of light [31]. The absorption spectroscopy technique uses this attenuation of light intensity, at a particular wavelength, after passing through a solution of an absorbing compound to measure the concentration of the particular compound [31].

UV-Vis spectrophotometry has been suggested as a ‘presumptive test’ for the preliminary identification of blood [84], and more recently, saliva [85]. In the case of saliva, the reaction was between a commercially available reagent and salivary amylase. A positive reaction was indicated by the formation of a yellow colour and therefore, absorbance was measured at 403 nm [85]. By using a UV-vis spectrophotometer to measure the absorbance of samples at 403 nm when treated with the saliva identification reagent, it could be possible to discriminate between human saliva and other fluids. The UV-vis spectrophotometer is able to scan the absorbance spectrum of a colour reaction. This will determine the peak absorbance of the

colour of the reaction, thus making this apparatus more sensitive than merely a visual confirmation of colour change.

Reena *et al.* (2014) showed that there was a difference in the reaction rate (i.e. the time the enzyme takes to generate a product) between saliva from different animals as well as different bodily fluids, when compared with the rate of human saliva [85]. This suggested that reaction kinetics could be a method for the presumptive qualitative differentiation of bodily fluid between humans and animals for forensic investigation.

Currently it is not known if there is any research into the use of UV-vis spectrophotometry to aid in the presumptive testing of semen. As previously described, the reaction of α -naphthol (the product of seminal AP activity) and brentamine FB (Figure 3) forms the purple azo dye which has been shown to absorb at 525 nm [13]. Taking advantage of this colour reaction, and if similar methods are derived from Reena *et al.*, (2014) UV-vis spectrophotometry can be an advantageous method to presumptively identify semen.

Confirmatory tests

An important subsequent step to confirm the source of the bodily stain is the use of a confirmatory test. For semen stains, this is usually in the form of sperm cytology. Cells are extracted from the stain using buffers and the resulting extracted solution, presumably containing sperm, is then mounted on a microscopic slide and stained with either haematoxylin and eosin or nuclear fast red and picroindigocarmine 'Christmas tree' stain [20–22]. If a characteristic sperm cell with a tail is observed, then it is confirmed that semen is indeed present.

The slides are also scored to determine the number of sperm cells found and if they contain tails. The qualitative scoring of the slides is indicative of the frequency of spermatozoa seen in the microscopic view and is denoted using symbols: hard to find spermatozoa (+), some spermatozoa in the microscopic view, easy to find (++), many or some spermatozoa in most views (+++) and many spermatozoa in all views (++++). In addition to scoring with the plus symbols, the letter T is used to denote the presence of tails on the spermatozoa seen on the slides [14,86]. This is used to determine the approximate time in which the semen stains were deposited and to draw time inferences for the crime and/or scene [23].

However, assessment of sperm cytology as a confirmatory test was performed by Tobe *et al.* (2015), who demonstrated a lack of consensus on the scores of 37 forensic scientists scoring the same slides [24], suggesting that this test is highly subjective. The absence of intact tails on the spermatozoa is indicative of a aged stain where spermatozoa has degraded [23]. As previously shown in table 1, the tail is one of the discriminating features of the spermatozoa between mammals, and if the tail is missing it would be difficult to discern the origin of the cell [23,40].

A study using the “Christmas tree” stain has shown to be able to distinguish human spermatozoa from other mammals [25]. The “Christmas tree” stain differentially stained the head and tails of spermatozoa, allowing for better observation of spermatozoon structure. The morphological differences between spermatozoa became more apparent. As seen in table 1, morphometric differences between the heads and tails of spermatozoa from different species have been previously observed.

Genetic profiling has also been used as a form of confirmatory test for semen [20,26–28]. Testing was performed on samples where the presence of sperm cells was confirmed through sperm cytology, it was also confirmed that male-specific DNA can be identified from stains which are absent of spermatozoa [10,11]. The DNA analysis targets the Y-chromosome, specifically the microsatellites on the chromosome. Y-chromosomal DNA can be obtained from male epithelial cells (surface skin cells) but these are differentially excluded during the tests on semen [26]. However Y-chromosome DNA determination on vasectomized males still yield a positive result meaning there are other contributors of the DNA [10]. This means that the test for Y-chromosomal DNA is not fluid-specific but rather determines if male DNA is present. This is a limitation of this ‘confirmatory test’, as it does not test for semen or any fluid at all, but rather male DNA in general, which could be from epithelial cells and not semen. Y-chromosome determination is also used in touching offences (sexual assault) and this can yield positive results from touch DNA (DNA left by transfer of epithelial cells) [10]. Y-chromosome DNA analysis has been used however, in the determination of identity in nonsexual offences [87].

There are a few commercial kits that are optimised to identify stains made by bodily fluids. The Rapid Identification of Stains (RSID™) (Independent Forensics, Illinois, USA) manufacture kits that test for urine, blood, saliva and semen. These tests use antibodies to fluid-specific antigens [88]. The RSID-Semen kit determines the present of Sg in the possible semen stain. This test is an ELISA membrane test which has been shown to be more sensitive than the test for PSA [28]. The test kit for Sg showed species-specific reactions with human semen when tested against semen from other mammals. showing a limit of detection at 2.5 nL of semen [9,28]. The RSID-Semen kit has been validated for forensic use [9,89].

There are several other commercially available kits that detect human PSA: ABACard® PSA test (Abacus Diagnostics California, USA), SERATEC® PSA Semiquant and SMITEST PSA card (Seratec, Göttingen, Germany). These kits also make use of antibodies that react with PSA. Validation studies of the ABACard PSA showed semen specificity when tested against other human bodily fluids but was unable to detect semen in post coital vaginal swabs after 24 hours after sex [74]. Laffan *et al*, (2011) showed that the SERATEC® PSA test kit had a 3% rate of false positive results [28]. The limit of detection for PSA using the SERATEC kit was shown to be a concentration of 200 ng/mL of semen [28].

A validation study on the commercial PSA detection kits determined that PSA was still detectable when the sample was subjected to temperature from 80 °C to 140 °C for one hour [90]. The SMITEST PSA kit was able to detect semen in a sample was diluted 1 in 10000 [90]. A comparison study of the ABACard p30 (PSA) kit and the RSID™-Semen kits showed that the RSID™-semen kit was unable to detect semen in post-coital vaginal swabs after 12 hour post sex whereas the ABACard p30 (PSA) kit could detect semen at 33 hour after sex [68].

Emerging techniques

The confirmatory identification of fluids is moving to molecular-based testing using RNA, DNA and epigenetics as biomarkers for differentiation [1,91–94]. Messenger RNA (mRNA) has been shown to be a biomarker which can distinguish between different bodily fluids and has been used in a proposed multiplex real-time PCR assay to profile bodily fluids [95,96].

The amount of DNA is relatively constant in cells, but the levels of mRNA vary due to tissue-specific gene expression. This means that different bodily fluids would have different levels of mRNA production which could be used as a profile, or fluid-specific mRNA transcripts could be used to discern between the different bodily fluids.

MicroRNAs (20 – 25 nucleotide in length), which are non-coding RNA molecules, have also been shown to be suitable biomarkers in identifying bodily fluids. MicroRNAs were shown to be fluid specific and have been shown to remain intact over time [97,98], making them ideal for forensic determination of bodily fluids. A study reported nine microRNAs as possible viable biomarkers, as they are variable enough between bodily fluids to identify specific fluids, and are able to detect the fluid type using a minimum of 50 pg of DNA [99]. A study by van der Meer *et al.* (2013) has shown that it is possible to include microRNA analysis of bodily fluid in the standard forensic DNA profile analysis with minimal modifications [93]. The forensic scientists showed the simultaneous extraction and analysis of microRNAs and DNA was possible, which results in a single electropherogram where the DNA profile generated from the bodily fluid and the identification of the bodily fluids can be obtained [93]

In general, RNA molecules are highly stable molecules and validation of these RNA-based methods showed that both mRNA and microRNA are capable of being amplified after being exposed to room temperature for more than a year [97,100]. Investigations into the outdoor exposure of stains indicated that tissue specific mRNAs were detectable at 180 days but if the stains were exposed to precipitation the time frame shortens to only a few days [100]. There is also the potential to use the constant degradation rate of mRNA transcripts to determine the age of stains [101,102].

The analysis of the epigenome (chemical changes which occur on the genome) indicated that DNA methylation carries tissue specific traits and this can aid in the identification of bodily fluids [103,104]. DNA methylation occurs on different loci of the genome and 15 of such loci have been proposed to differentiate between different bodily fluids and skin epidermis [103]. As little as one nanogram of DNA can be used to identify the fluid; this is advantageous when the sample size in forensic cases can be minute [91,103]. Preliminary investigations into application of the DNA methylation analysis has shown it is compatible with the current forensic DNA profile assay [103].

These alternative techniques have the capability to be more effective than the current assays with regard to the identification of bodily fluids. They have been shown to be sensitive and specific. Their prospective inclusion into the current DNA profile assay is gainful as samples need not undergo additional presumptive or confirmatory testing. Although these emerging techniques show promise, they are not currently being used in forensic analysis as they have not been validated and integrated into current commercial forensic assays available.

Conclusion

Semen is one of the most abundant bodily fluids encountered in sexual assault cases. Semen is made up of spermatozoa and seminal fluid, where the latter includes several components, namely PSA, Sg and AP. These components, as well as spermatozoa, are forensically important as they are targets for the presumptive and confirmatory identification of semen. Of particular interest is AP which is an enzyme found in high concentration in semen. The presumptive forensic test for semen exploits the AP enzymatic capability by using a reagent that, when altered by AP, changes colour. Different forensic laboratories around the world use different cut-off times to exclude possible false positives. In South Africa the cut-off is 65 seconds and additionally, semen is presumed to be from human origin if it reacts within 50 seconds; however it is unclear what research has been performed, if any, to substantiate the use of these cut-off values in South Africa.

The varied, and somewhat arbitrary, cut-off times for the AP reaction advocates a more objective measurement of a visual colour change, and spectrophotometry could be one consideration. UV-Vis spectroscopy can objectively measure a change in colour and the time of the reaction to generate the reaction rate, or kinetics, of the reaction. This could be a more accurate method than merely a visual detection and manual timing of a colour change. The activity of AP has been shown to vary between mammals and the reaction rate of the enzyme perhaps could be used to differentiate between specimens; however, this has not yet been studied for AP.

Another method that could be used to differentiate between human and animal samples is the use of confirmatory tests for semen (microscopy), where studies have reported morphological differences between the spermatozoa of different mammals.

The immunological tests for semen are have been shown to be more sensitive and specific, than the enzymatic tests. However, different brands of these tests have shown varied degrees of sensitivity. The emerging molecular techniques being developed for fluid identification has the potential to be the most sensitive methods. Studies showing the compatibility of the molecular with the DNA profile assays indicate moving toward a more comprehensive and inclusive method. This would involve less of the evidentiary samples being consumed for identification purposes. Investigations into the permanence of microRNAs in old stains alludes to the probable benefit to help solving cold cases.

The identification of bodily fluids is vital to understanding the sequence of events that led up to the crime, and to the possible identification of the perpetrator. Advancements and additional techniques can enhance the information gleam from evidentiary stains and bodily fluids. Although these innovative techniques have not been validated for forensics yet, they their inclusion into the forensic field will improve the quality of information obtained from bodily fluids.

Part C. Publication- ready Manuscript

Investigation into presumptive and confirmatory testing of semen for use in forensic science

Abstract

The identification of bodily fluids for forensic purposes is typically classified as either presumptive or confirmatory. Presumptive tests (PT) are conducted first to screen for certain compounds which are relatively specific to particular fluids. Confirmatory tests are used to confirm the identity of a body fluid. Semen is one of the most common bodily fluids encountered in sexual assault cases and contains high concentrations of the acid phosphatase (AP) enzyme. The brentamine FB reagent reacts with the AP that is present in semen, and turns purple. If the colour change is observed within a specific time threshold, it is considered presumptively positive for semen. Cut-off time varies considerably between forensic laboratories, but in South Africa, the cut-off time is defined as 65 seconds. Additionally, semen may be considered to be from human origin if it reacts within 50 seconds. These cut-off times have been arbitrarily defined, and there is little research in a local context to substantiate or inform the threshold time for the brentamine FB test for semen. Therefore this study assessed the sensitivity, specificity and kinetics of the brentamine FB test on semen from South African male volunteers (n=15), canines (n=2) and various fruit extracts and compared these results to purified human AP. Each semen sample was subjected to the PT in an indirect and direct method, and these tests were performed both on fresh and aged samples. The majority of fruit extracts yielded a distinctly different colour change compared to the purple that was produced from semen except for mushroom which also turned purple. Absorbance spectroscopy was used to determine the rate of the reaction at 525 nm. There were no significant differences between the rate of reaction for fresh and aged samples using both direct and indirect testing. All undiluted specimens (human, canine and fruit extracts) reached saturation within 50 seconds, which highlights the need to redefine the cut-off times for the brentamine FB test for semen. Furthermore, this study demonstrates the potential utility of absorbance spectroscopy as a novel method for presumptive testing of semen and may have implications for methodology used in forensic laboratories. The confirmatory test for semen typically conducted examines sperm cytology by haematoxylin and eosin staining, which we performed on semen stains from human and canine samples. Morphometric differences in spermatozoa head shape were observed. Thus, although the brentamine FB test

could not discern between human and canine samples, sperm cytology as well as absorbance spectroscopy both hold potential for species differentiation between human and canines.

Keywords: acid phosphatase, brentamine FB, UV-Vis spectroscopy, sperm cytology, canine, semen, kinetics

1 **Introduction**

In cases of sexual assault, the location and identification of semen is essential in the forensic examination. The water soluble enzyme, acid phosphatase (AP), is produced in the testes and found in high concentrations in the semen of mammals [1,3,42]. The presumptive test for semen, namely Brentamine Fast Blue (FB) reagent, tests for AP in possible stains. Such presumptive tests make use of a chemical reagent that changes colour in the presence of AP [8,105]. This reagent consists of brentamine FB and α -naphthyl phosphate [12]. The AP in semen hydrolyses the phosphate group on α -naphthyl phosphate converting it to α -naphthol. The α -naphthol then binds to the brentamine FB which results in the formation of a purple azo-dye [12]. This colour reaction develops over time; if the colour reaction is observed within a specific threshold time, the stain is considered presumptively positive for semen. [3,12].

Presumptive tests for semen are quick and easy to perform at the crime scene or in the laboratory [65–67]. The test for AP, however, is not specific for semen, as there are other substances that contain AP and can thus generate a ‘false’ colour change [3,17,72,105]. The non-specificity of the brentamine FB test has prompted forensic laboratories to use a cut-off time for the reaction. Specifically, if the stain develops a colour change within a certain period of time after application of the reagent, it is considered presumptively positive for semen [19,73,74]. However, this time varies according to region. In Spain, the United Kingdom, Canada, India and South Africa, a cut-off time of 300, 120, 90, 60 and 65 seconds are used, respectively [12–15, South African Police Services Forensic Science Laboratory Standard Operating Procedure]. Additionally, during a court case in South Africa, a forensic scientist testified that the presumptive test for semen yielded a positive result in 50 seconds, and therefore the semen stain was considered to be from human origin. The transcript of this case was not available for review, but the testimony nevertheless posed a complication going forward, in that it was suggested a presumptive test can be used to differentiate between human and animal semen.

Enzymatic activity is defined as the rate or velocity of an enzyme, which is the amount of the particular product formed per second. Research into the enzymatic activity of AP from semen has shown that human AP has an activity range of 250 – 1300 U/mL of semen [77,78,80]. Of note, investigations into the activities of animal AP in semen has

shown that the enzymatic activity was relatively lower than that of humans; 24.7 UI for bulls [81], 64.9 UI for rams [82], 680 UI for pig [83] 11.5 UI for donkeys [80] and 1820.3 U/L for dogs [106]. Therefore, if animal semen were to be tested using the presumptive brentamine FB test, the reaction may take longer compared to semen from humans. If the animal semen stains were to develop after the cut-off time, the animal samples would then be considered negative.

Non-semen substances (e.g. tea and fruit extracts) and other bodily fluids (e.g. vaginal fluid) have also been reported to react positively with the brentamine FB test, albeit some exhibited a different colour change and took a relatively longer time to develop a colour change [3,17]. However, the brentamine FB test may have large variations in specificity and sensitivity given there is no consensus on cut-off times. The existing cut-off times have not been rigorously tested, with only a few studies reported, which have challenged the established cut-off times in certain countries [14,18,19]. Such limitations of the presumptive test for semen highlight the need for an improvement of the current presumptive test method, particularly in South Africa, or perhaps (re)defining and implementing better training programmes with regards to the interpretation of positive reactions within certain cut-off times in a South African context.

Ultraviolet-visible (UV-vis) spectrophotometry is a technique which can measure the intensity of colour reaction at a specific wavelength of light, and uses this to calculate the concentration of the product being generated [31]. Spectrophotometry may be used in the detection of bodily fluids. Indeed, UV-vis spectrophotometry has previously been used to determine the presence of blood [84]. In the case of the brentamine FB test, the product is the purple azo dye which can be measured at a wavelength 525 nm [13].

A confirmatory tests for semen is the microscopic determination of the presence of spermatozoa. Slides are differentially stained with haematoxylin and eosin or nuclear fast red and picroindigocarmine (Christmas tree stain) and are scored according the amount of spermatozoa seen and the presence of tails [20–23,86]. The presence of intact, attached tails has been reported as an indicator of time since deposition of semen [86]. Boward *et al.* (2015) showed that the ‘Christmas tree stain’ can be used to distinguish between the spermatozoa of different mammals [25], as morphological differences were observed. These included the length of the cell and the shape of the sperm head which can be used in morphometric analysis [40].

Here, we investigated the sensitivity, specificity and reaction kinetics of the brentamine FB test in a South African context, in order to assess the existing cut-off time of 65 seconds. We also assessed the 50 seconds cut-off time to distinguish between human and canine semen stains. The use of UV-Vis spectrophotometry and enzyme kinetics in presumptively identifying semen was investigated and the method of haematoxylin and eosin staining of spermatozoa of human and a non-human mammal in order to discern morphological differences was evaluated

2 Materials and methods

2.1 Samples

2.1.1 Human

Participants gave informed consent and the study received ethics approval by the University of Cape Town, Faculty of Health Science, Human Research Ethics Committee (HREC 502/2015). Participants were approached and given information, and the donation schedule was planned which was suitable to them. The participants were asked to abstain from any ejaculations for 36 hours before donations and to donate in the mornings. Two samples were then self-collected by the volunteer into 50 mL sterile tubes, the second sample was donated two weeks after the first. In total, semen samples (approximately 5 mL) were obtained from fifteen unrelated male adult volunteers.

2.1.2 Non-human

Semen samples were obtained from two separate, unrelated male canines. One donation from each canine was obtained.

Extracts were made from different food produce, this includes: mushroom, red and white grapes, apple, and tea (i.e. Rooibos and Ceylon tea). Samples were extracted from the produce using a cold press technique, where the produce was squeezed and the liquid was collected. The tea samples were made as follows: one tea bag was placed in 180 mL of boiling water and allowed to brew for 3 minutes. The tea bag was then removed and the tea was allowed to cool.

2.2 *Preparation of the brentamine FB reagent*

The brentamine FB reagent was prepared by combining 2 stock solutions (solution A and solution B). Solution A was prepared by dissolving 2 g of sodium acetate and 0.1 g of *o*-Dianisidine (Sigma-Aldrich, Germany) in 10 mL of deionised water and 1 mL of glacial acetic acid. Solution B was prepared by dissolving 0.4 g of sodium α -naphthyl phosphate (Sigma-Aldrich, Germany) in 5 mL of deionised water.

The brentamine FB reagent was prepared by combining 5 mL of solution A, 0.5 mL of solution B and 44.5 mL of deionised water. Solution A and the AP reagent were made fresh on each day of testing.

2.3 *Sensitivity*

A series of dilutions (1 in 4, 1 in 10, 1 in 40, 1 in 100, 1 in 400, 1 in 1000, 1 in 2000 and 1 in 4000) were prepared from fresh semen samples using deionised water. The semen dilutions were mixed using a vortex. A volume of 200 μ L of each dilution, as well as neat semen, was seeded onto separate sections (7 cm x 7 cm) of white poly-cotton fabric, in duplicate. The fabric was allowed to air-dry for one hour then packaged in plastic re-sealable bags. The first semen donation were left at 22 °C for two weeks until the second donation was made. The second donation was diluted as seeded on to fabric and air dried as explained previously. The first donation (aged specimens) was then tested (as described in 2.3.1 and 2.3.2) simultaneously with the second donation (fresh specimens), on the day the second donation was made.

A series of dilutions of purified human acid phosphatase (Merck, Germany) (600 U/mL, 300 U/mL, 100 U/mL, 50 U/mL, 20 U/mL, 10 U/mL, 5 U/mL and 2.5 U/mL) were prepared in deionised water. These dilutions were seeded onto poly-cotton fabric as described above and allowed to air-dry at room temperature for one hour.

2.3.1 *Press method and indirect testing*

Before direct testing was performed Whatman Grade 1 filter paper circles (Whatman, UK) were moistened with 200 μ L of deionised water, and were place on top of the seeded

fabric. Constant pressure was then applied over the filter paper for five seconds. The filter paper was then treated with the brentamine FB reagent and the reaction was timed, in seconds (s), until a colour change was observed.

2.3.2 Direct testing

A volume of 50 μ L of the brentamine FB reagent was applied to the side of the fabric containing the semen or purified human AP, and the time for a purple colour to be observed on the fabric was recorded. If there was no reaction after 10 minutes after treating with brentamine FB, the specimen was considered negative.

2.4 Specificity

A series of dilutions were prepared, as described in section 2.3, of the canine samples in duplicate for each donation, as there was only one donation made by each canine. The canine samples were then processed in the same manner as the human samples (2.3.1 and 2.3.2).

A series of dilutions (neat and 1 in 2) were made of the food extracts, and processed as the human samples (2.3.1 and 2.3.2), but in triplicate. The colour change after AP reagent treatment of the food extracts was compared to the colour change of fresh undiluted human semen (positive control) and water (negative control).

2.5 UV-vis spectrophotometry

2.5.1 Semen specimens

A series of dilutions (1 in 100, 1 in 200, 1 in 300, 1 in 400, 1 in 500, 1 in 600, 1 in 800 and 1 in 1000) were made using fresh semen samples and samples that were left at room temperature for 2 weeks (aged).

2.5.2 Purified AP and food extracts

A series of dilutions of purified human acid phosphatase (Merck, Germany) (10 U/mL, 5 U/mL, 2.5 U/mL, 1 U/mL, 0.5 U/mL, 0.25 U/mL, 0.125 U/mL and 0.0625 U/mL)

were prepared in deionised water. Dilutions (1 in 10, 1 in 20, 1 in 50, 1 in 80) of the food extracts mentioned in section 2.4 were prepared in deionised water.

2.5.3 Reaction rates

A volume of 800 μL of the sample dilution was placed into a 2 mL plastic cuvette. The cuvette was then placed in a Shimadzu UV1800 (Shimadzu, Japan) spectrophotometer and 200 μL of the AP reagent was added. The UVProbe 2.3.3 software (Shimadzu, Japan) was used to acquire the absorbance of the reaction at 1 second intervals, at a wavelength of 525 nm for two minutes.

2.5.4 Reaction rate calculations

The acquired absorbance reading for each sample was exported to Microsoft Excel (Microsoft, USA) where the data was plotted as a line graph. The best-fit trend-line was established for the exponential phase of the reaction and the slope (m) of the trend-line equation ($y = mx + c$) was taken as the rate of the reaction.

2.5.5 Absorbance Spectrum

The absorbance spectrum of sample reacting with brentamine FB was determined. The Shimadzu UV1800 (Shimadzu, Japan) spectrophotometer was set to spectrum mode and the 1 in 200 dilution factor for the semen and the neat food extract specimens were used. A volume of 800 μL of the sample was placed into a 2 mL plastic cuvette. The cuvette was then placed into the spectrophotometer and 200 μL of the AP reagent was added. The reaction was allowed to develop for 5 seconds, using the UVProbe 2.3.3 software (Shimadzu, Japan) an absorbance reading was then acquired at every wavelength of light between 450 – 700 nm for each specimen.

2.6 Statistics

The number of biological repeats ($n=15$) was the maximum number of samples that could be analysed within the scope and timeframe of the study. The analysis of these samples

was anticipated to give a general idea of the biological variance of enzyme activity of AP. This would help us estimate the ideal cut-off.

The non-parametric Wilcoxon matched pair statistical test was performed on the reaction time determined in 2.3 and 2.4, and the reaction rates determined in 2.5.4 using Prism 5 (Graphpad, USA). This statistical test was chosen as the sample size was small ($n = 15$) and the data was not normally distributed. A p-value of less than 0.05 was considered statistically significant.

2.7 *Spermatozoa extraction and staining*

Sections (1cm x 1cm) were cut from the middle of the stain in the fabric. Each section was placed in a 1.5 ml tube and submerged in 400 μ L of deionised water. The section was manually agitated for ten seconds using forceps and then vortexed for five seconds. The section was then squeezed using forceps and the section was discarded. The tube was centrifuged for one minute at 13,000 g. The supernatant was discarded and the pellet was re-suspended in 20 μ L of deionised water. A volume of 5 μ L of the extract was mounted onto a Histobond® slide (Marienfeld, Germany) and allowed to dry. The slides were placed in a Tissud-Tek® Prisma Automated Slide Stainer (Sakura, Japan) and stained using haematoxylin and eosin. The slides were subsequently moved to a Tissud-Tek® Glas™ Coverslipper and mounted with coverslips before being examined under a microscope. All slides were examined under 400x magnification using a Leica DM500 compound microscope (Leica Microsystems, Switzerland) mounted with a camera.

The qualitative scoring of the slides was carried out by evaluating the frequency of spermatozoa seen in the microscopic view, as follows: no spermatozoa (0), hard to find spermatozoa (+), some spermatozoa in the microscopic view, easy to find (++), many or some spermatozoa in most views (+++) and many spermatozoa in all views (++++). [23]. The median score for each dilution factor was determined. The number of observed slides where tails were attached to sperm heads was tallied. Photographs were taken and processed using Leica Application Suite LAS EZ version 3 software (Leica Microsystems, Switzerland). Four spermatozoa heads were chosen at random from neat, fresh human and canine semen extractions, and the mean spermatozoa morphometric parameters were determined. Statistical

testing was performed using Prism 5 (Graphpad, USA). Non-parametric Mann Whitney test were used and a p-value of less than 0.05 was considered significant.

3 Results

3.1 Sensitivity

By studying the time taken for the specimens to react different testing methods, we can infer that direct testing is the more sensitive method. Figure 1 illustrates that the direct application of the brentamine FB test was more sensitive than the indirect test for human semen. There were statistically significant differences between direct and indirect testing for fresh specimens. Specifically, direct testing reacted faster than indirect for neat ($p=0.0011$), 1 in 4 ($p=0.0011$), 1 in 10 ($p=0.0011$) and 1 in 40 ($p=0.0156$; Figure S1). This was also seen when testing direct and indirect reactions for aged samples, with the neat, 1 in 4 and 1 in 10 dilution factor having a p-value of 0.0007 and 1 in 40 having a p-value of 0.0091 (Figure S1). There was no significant difference found for the 1 in 100 dilution for either fresh or aged specimens (Table S1).

Figure 1A-D shows an increasing trend in reaction time as the dilution factor of semen increases, for both direct and indirect testing. The mean reaction times for neat specimens for both fresh and aged semen stains when tested either directly or indirectly, was less than 10 seconds (fresh direct = 2 ± 0.98 seconds (s); indirect = 8.14 ± 5.30 s and aged direct = 2.5 ± 1.35 s; indirect = 6.93 ± 1.98 s) (Table S1). There was no statistically significant difference between fresh and aged semen samples at any dilution factor, for both direct and indirect tests. Figure 1B, D and F show a loss of sensitivity, i.e. there was no positive reaction within 10 minutes. For indirect testing, the 1 in 100 dilution factor for the fresh specimens had mean reaction time of 214.33 ± 55.82 s and 238 ± 87.68 s for aged specimens (Table S1). Whereas for direct testing, positive reactions were detected for fresh ($n = 3$) and aged ($n = 2$) specimens at 1 in 4000 dilution factor, with a mean reaction time of 240 ± 120.7 s and 355 ± 77.78 , respectively (Figure 1E, Table S1). Fresh specimens from one participant did not react at any concentration when tested indirectly (Figure 1F).

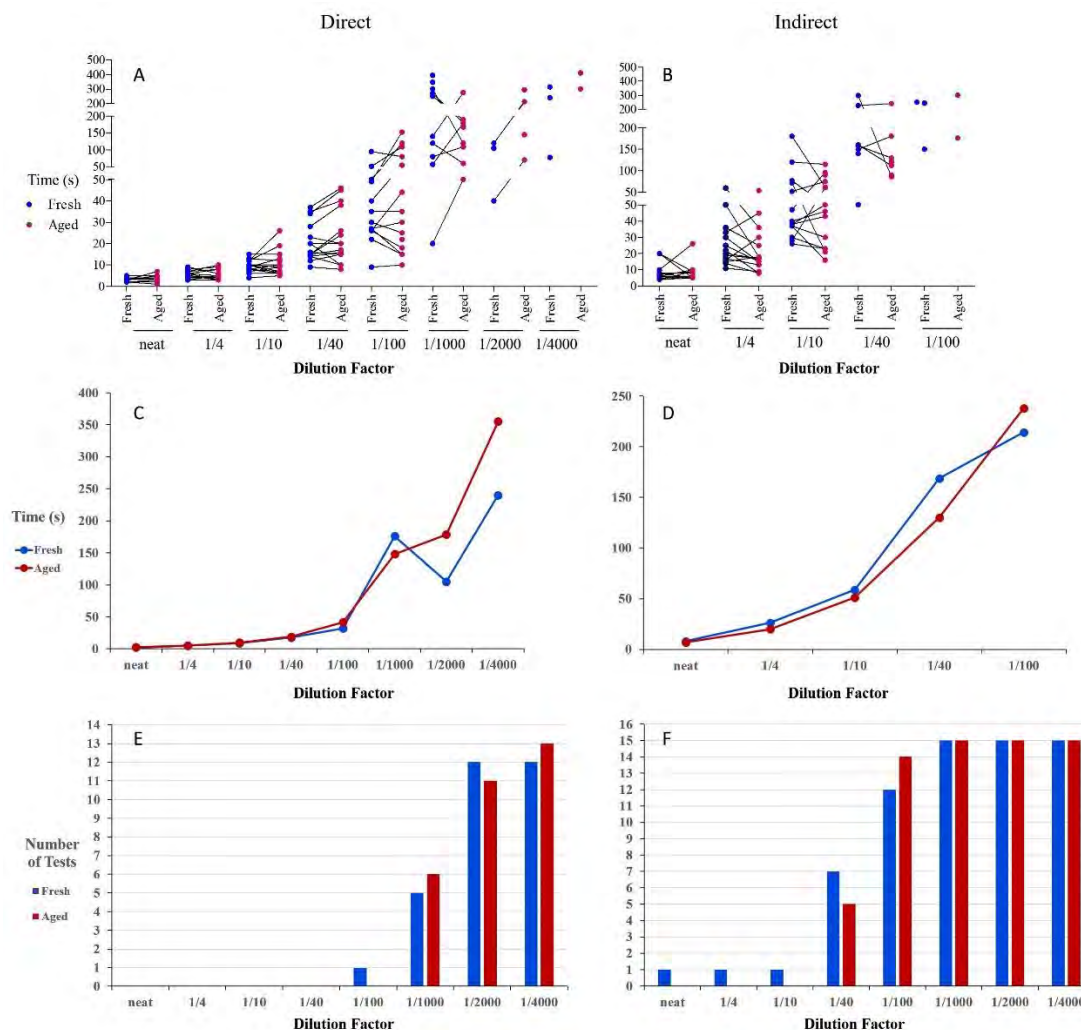


Figure 1: The direct and indirect testing of human semen stained onto fabric using the brentamine FB test (n = 15). A (direct) and B (indirect), show the reaction times of fresh semen stains (blue dots) and the paired aged semen stain (red dot) for each dilution factor of semen sample for each participant. A and B, only the samples which reacted positively were plotted. Statistical comparisons were performed using a non-parametric Wilcoxon paired test. C (direct) and D (indirect) illustrate the mean reaction times for each dilutions factor for fresh (blue line) and aged (red line) semen stains. The bar graphs E (direct) and F (indirect) show the number of non-reactive tests for each dilution of fresh (blue bars) and aged (red bars) semen stains.

Direct testing was performed on decreasing concentrations of purified human AP to determine the limit of detection of the brentamine test, as well as a quantitative comparison to semen. The reaction time for purified AP increased as the concentration of enzyme decreased (Figure 2). Enzyme at a concentration of 600 U/mL reacted positively in 5 s, which is comparable to the reaction time of the 1 in 4 dilution of directly tested fresh or aged specimens (Table S1). There was no positive reaction for purified AP at an enzyme

concentration of 2.5 U/mL when directly tested. The concentration of 5 U/mL was considered the limit of detection and the reaction time was recorded as 177 s. This was similar to the reaction times of the 1 in 1000 and 1 in 2000 dilution factors for the aged and fresh directly tested specimens (Table S1).

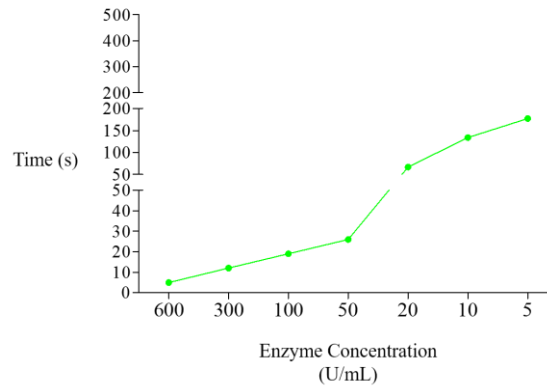


Figure 2: Reaction time of purified human AP when testing directly using the brentamine FB test.

3.2 Specificity

As previously seen with human semen, canine semen exhibited an increase in reaction time for both direct and indirect testing as the dilution factor increased (Figure 3). There was also a loss in sensitivity after the 1 in 10 and 1 in 40 dilution factor for aged and fresh canine specimens, respectively, when tested directly (Figure 3A and C). The fresh and aged neat semen specimens reacted positively at 18.50 ± 2.12 s and 69 ± 38.18 s, respectively (Table S1), which was slower than the directly tested neat human specimens (Table S1). The reaction time for the fresh, 1 in 40 dilution factor canine specimen was 210 s, which was also slower compared to the same dilution factor for the human specimens. Furthermore, the aged, 1 in 10 diluted canine specimens reacted at 239.5 ± 113.84 s (Table S1).

The indirect testing of canine semen showed loss of sensitivity at the 1 in 10 dilution factor for the fresh specimens but for aged specimens only the neat semen gave a positive reaction (Figure 3B and D). The reaction time for the neat, fresh specimens was 36 ± 8.49 s, which was in fact comparable to the 1 in 4 dilution factor for indirectly tested fresh human semen specimens. The neat, aged canine specimens reacted in 240 ± 42.43 s which was similar to the 1 in 100 dilution of the human specimens. Thus, these results demonstrate that

canine semen specimens do give a positive colour change but react considerably slower than human specimens at the same dilutions and conditions.

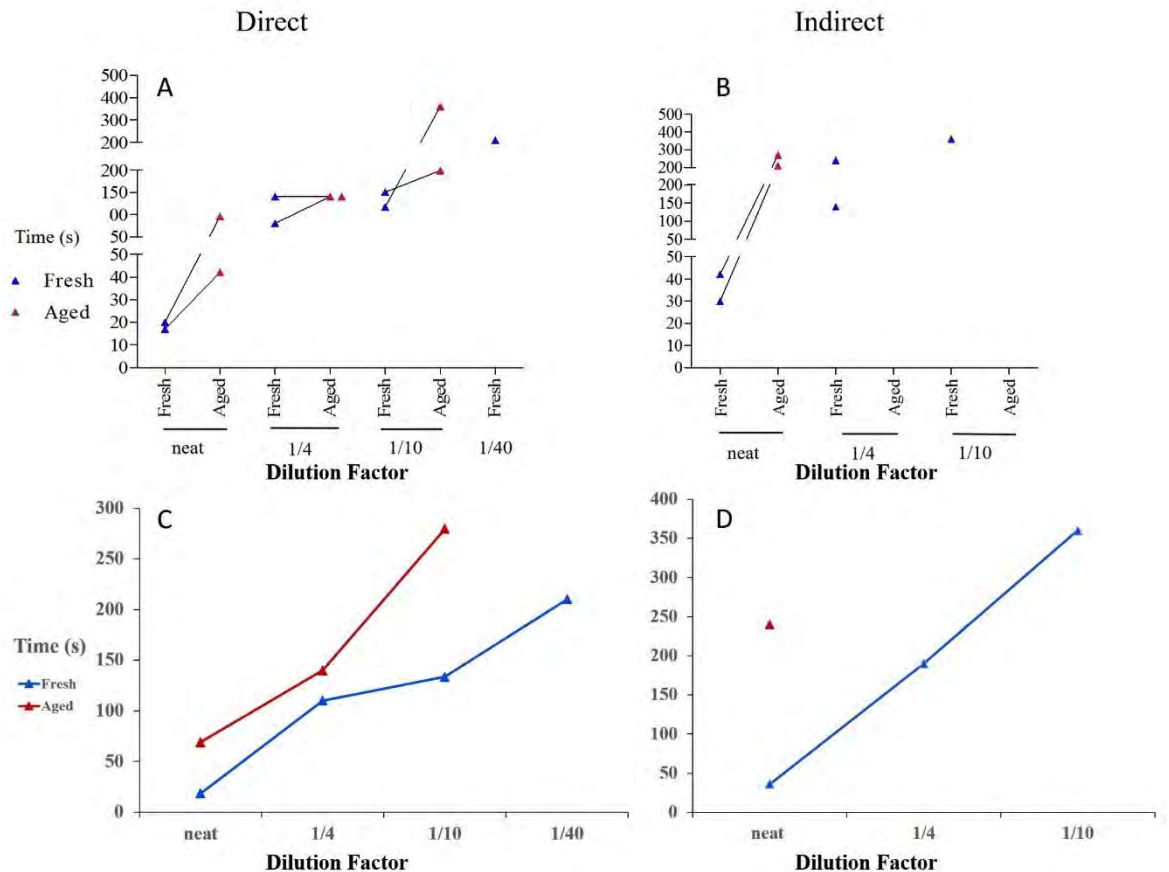


Figure 3: The direct (A and C) and indirect (B and D) testing of canine semen using the brentamine FB test (n = 2). A (direct) and B (indirect), show the reaction times of fresh semen stains (blue triangles) and the paired aged semen stain (red triangles) for each dilution factor of semen sample for each specimen. C (direct) and D (indirect) illustrate the mean reaction times for each dilution of fresh (blue line) and aged (red line) semen stains.

The reaction times for the non-semen samples was then investigated. Figure 4 shows the reaction times for the food extracts. Ceylon tea reacted the quickest, (within 2 s) while Rooibos tea reacted relatively slower 40.67 ± 4.51 s. The reaction time for the rest of the neat food extracts was less than 50 s, which is also within the cut-off time for semen (Figure 5; Table S1), thus these specimens reacted within the cut-off for human semen. The direct and indirect testing of aged canine semen and the purified AP enzyme concentration of 20 U/mL reacted positively after 65 seconds (Figure 5).

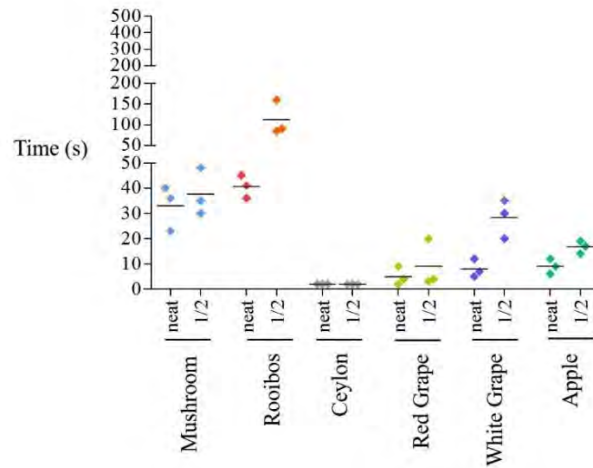


Figure 4 The reaction times of fabric stained with food extracts using the brentamine FB test directly (n=3): mushroom (blue), Rooibos tea (orange), Ceylon tea (grey), red grape (yellow), white grape (purple) and apple (green) when tested using the brentamine FB reagent.

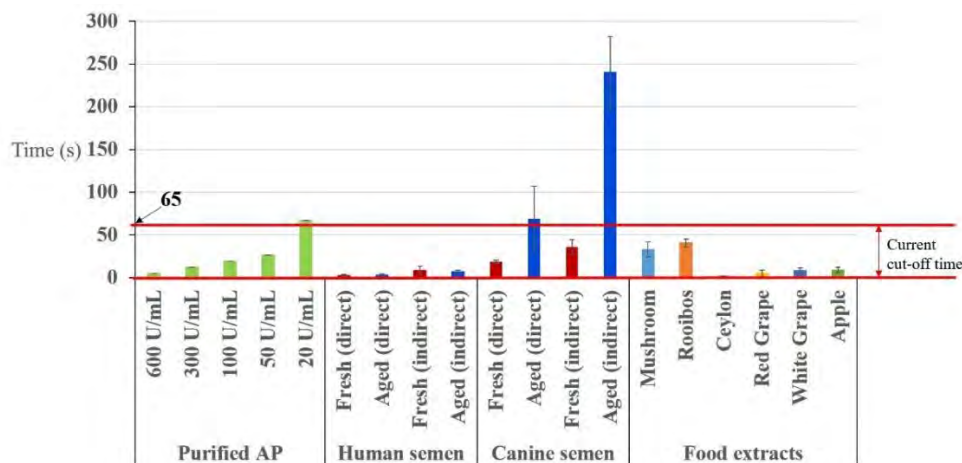


Figure 5: The reaction times for dilutions of purified AP and neat stains for each specimen using the brentamine FB test. The red line indicates the 65 second cut-off time for presumptive positive identification of semen.

3.3 UV-vis Spectrophotometry

3.3.1 Reaction rates

The reaction rates for all specimens decreased with increasing dilution factor (Figure 6). The reaction rates of human aged and fresh semen were statistically different at 1 in 100 ($p = 0.0302$) and 1 in 200 ($p = 0.0256$) dilution factors, whereas there were no differences for

the lower dilution factors (1 in 300 to 1 in 1000) (Figure S2). The mean reaction rates for human aged and fresh 1 in 100 diluted specimens was 0.2454 ± 0.1337 Abs/s and 0.1926 ± 0.0733 Abs/s, respectively (Table S2). The canine aged and fresh specimens for the same dilution factor had mean reaction times of 0.0144 ± 0.0039 Abs/s and 0.0162 ± 0.0057 Abs/s, respectively (Table S2). The reaction rate of 10 U/ml of purified AP was 0.2288 Abs/s. At a mean reaction rate of 0.1807 Abs/s, the rate of reaction of Ceylon tea was higher than the other food extracts (Table S2). Thus, human semen, purified AP and the higher dilutions of Ceylon tea have relatively higher reaction rates compared to other specimens tested (Figure 6).

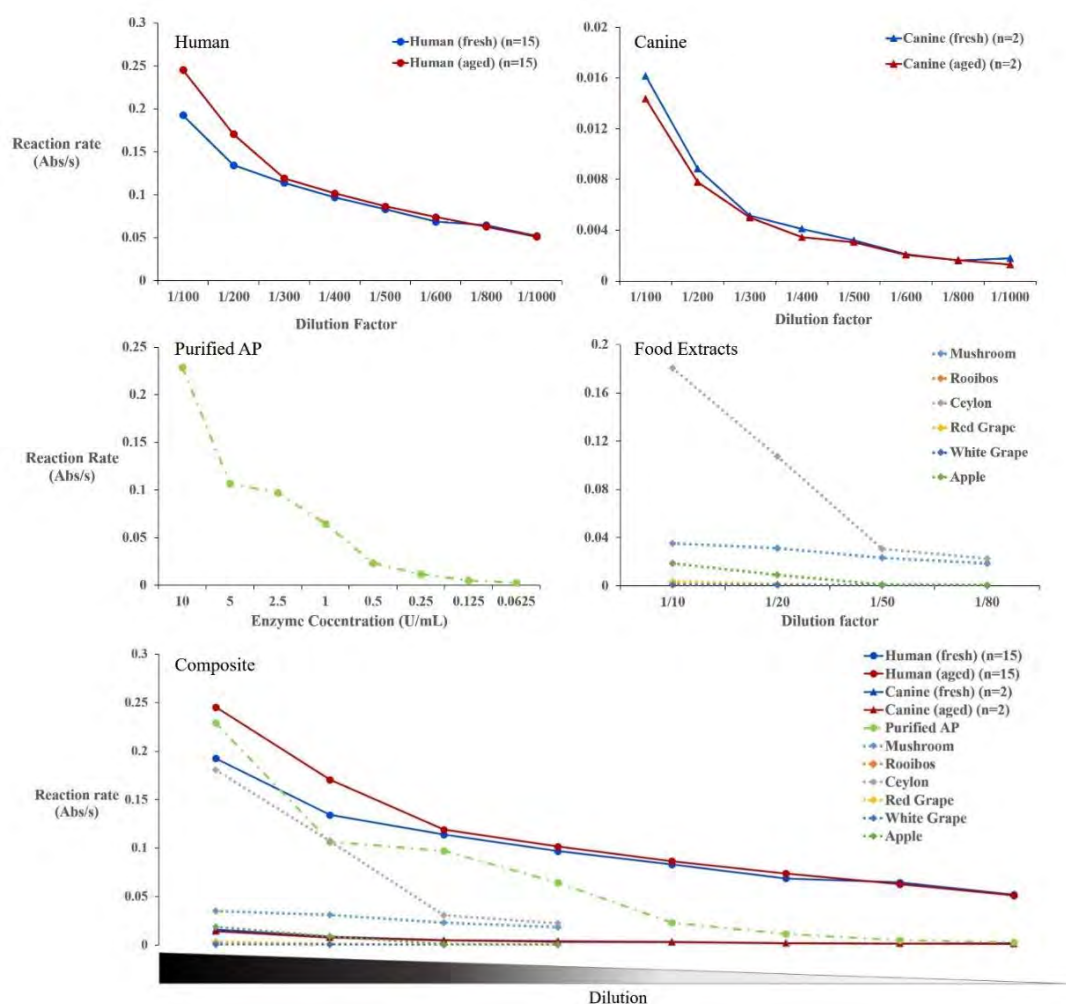


Figure 6: The curves of the reaction rates of AP reactions for different specimens using the brentamine FB test: fresh (blue circles) and aged (red circles) human semen, fresh (blue triangles) and aged (red triangles) canine semen, purified AP enzyme (green circles) and food extracts (diamonds): mushroom (light blue), Rooibos tea (orange), Ceylon tea (grey), red

grape (yellow), white grape (blue) and apple (green). The composite graph is not drawn to scale on the x-axis.

3.3.2 Absorbance spectrum

The absorbance spectrum for each specimen after brentamine FB testing was determine using UV-vis spectrophotometry. Table 2 shows the final colour, peak wavelength and absorbance for specimens. Water was used as a negative control and undiluted fresh human semen was used as the positive control. Of note, mushroom extract was the only non-semen extract with the same peak wavelength as semen, which is 513 nm, when reacting to brentamine FB. This was also observed when mushroom extract stains were directly test with brentamine FB (Figure S2A). The mushroom stain gave a purple colour, similar to the presumptive positive colour change for semen. The absorbance spectra for mushroom and semen have similar profiles (Figure S2B). Although the other food extracts react with brentamine FB, they do not develop the presumptive purple colour. (Table 2 and Figure S2A).

Table 2: The final colour, peak wavelength and absorbance measure for each specimen

Specimen	Final Colour	Peak wavelength (nm)	Absorbance
Water	colourless	450	0.13
Semen	purple	513	1.23
Mushroom	purple	513	2.71
Rooibos	light orange	450	1.68
Ceylon	dark orange	499	2.59
Red Grape	light orange	498	0.44
White Grape	light orange	498	0.62
Apple	light orange	450	1.42

3.4 Sperm Cytology

Slides mounted with extractions from neat, fresh and aged semen stains showed the highest median cytological scores with ++++ and +++, respectively. The extractions from neat samples also had the highest frequency of slides positive for spermatozoa (fresh: n = 12 and aged: n = 13) (Figure 7A). Both the cytological score and the number of spermatozoa positive slides decreased with the increasing dilution factor (Figure 7A). Despite having four

and six slides positive for spermatozoa from the 1 in 40 diluted fresh and aged semen stains, respectively, the overall median cytological score was zero. This is also seen for the 1 in 1000 dilution where there two spermatozoa positive slide for the fresh samples and five for the aged samples but there is a median cytological score of zero. As expected, the number of slides positive for sperm tails was the highest in the extractions from neat fresh semen stains ($n = 11$; Figure 7B). Tails were observed in the aged specimens but less frequently compared to the fresh specimens (Figure 7B). Intact tails were observed in the 1 in 100 dilution for fresh specimens, but none were seen in the aged specimens for the same dilution factor (Figure 7B).

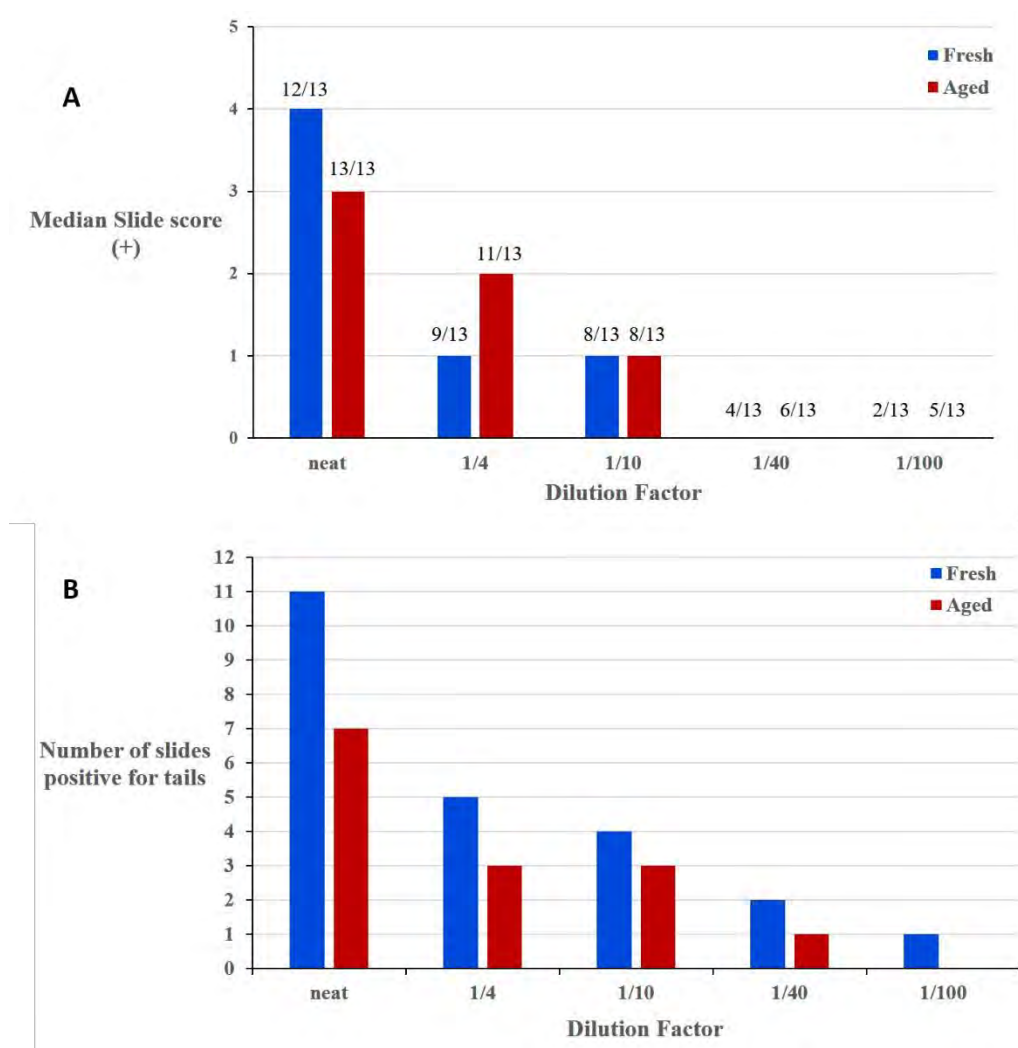


Figure 7 The median cytological score of the slides (A) and the number of slides positive for tails (B) for each dilution of fresh (blue) and aged (red) semen extracted from fabric ($n = 13$). In A the numbers appearing above each bar represent the number of slides which showed any presence spermatozoa.

Figure 8 shows spermatozoa extracted from fabric stained with human or canine semen. The haematoxylin staining of the canine sperm was more diffuse across the head, whereas for the human sperm there was a more intense haematoxylin staining from the middle to the posterior of the spermatozoon head (Figure 8 red arrow). The head of canine spermatozoon, which measured $7.68 \pm 0.29 \mu\text{m}$ in length and $5.08 \pm 0.31 \mu\text{m}$ in width, was larger than the human spermatozoon head which measured $5.28 \pm 0.43 \mu\text{m}$ and $4.02 \pm 0.31 \mu\text{m}$, in length and width respectively (Table S3). There were statistically significant ($p = 0.0286$) differences between the lengths and widths of the human and canine sperm head (data not shown). Extractions were previously done on semen stains that had been directly stained with brentamine FB resulting in some interference on the slide making it was difficult to distinguish any intact tails attached to the spermatozoa (Figure S5). The morphological differences can be seen between spermatozoa extracted from semen stains from different species of mammal when using haematoxylin and eosin histological staining.

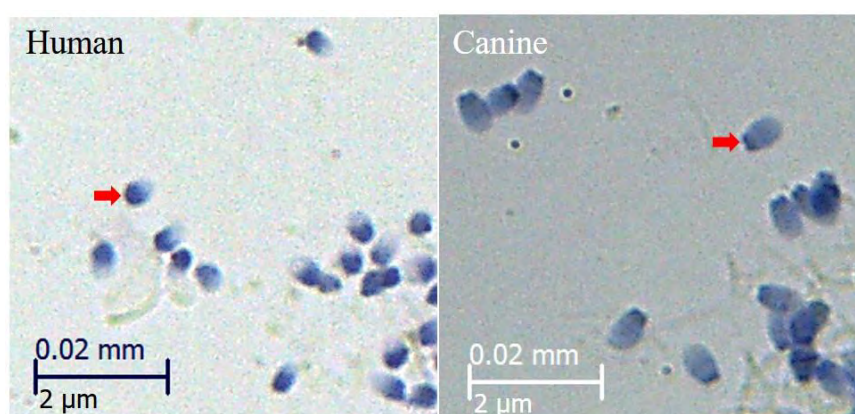


Figure 8: Haematoxylin and eosin stained spermatozoa extracted from fabric seeded with human semen and fabric with canine semen, with scale bar indicated. The red arrow indicates the intensity of the haematoxylin staining of the spermatozoon head.

4 Discussion

After testing the sensitivity of the brentamine FB test on human semen, the direct method appeared to be the more sensitive for detecting semen in both fresh and aged samples, with positive reactions occurring at the 1 in 4000 dilution factor. This is not surprising as there is the stain does not get diluted when using the indirect method [3]. However, the reaction times for the more dilute samples fell outside the cut-off for a presumptive positive

result (reaction time of less than 65 s), indicating that the stain would have been recorded as negative for semen. If we define sensitivity as the lowest dilution which reacts within the cut-off time, then 1 in 100 dilution is more suitable measure of sensitivity, as it is the most dilute specimen which reacts within this time. The more common indirect method was able to detect semen up to a dilution factor of 1 in 100 but the 1 in 10 dilution factor was the limit of detection for a presumptive positive using this method of testing.

Studies into the sensitivity of AP testing have shown varied results. In Spain the cut-off for presumptive positive for AP is 5 minutes and a study showed that their test was able to detect AP in the a dilution of 1 in 25,000 of fresh semen from a control subject [10]. However, their methodology, which was not described, may have differed from the method used in this study. Studies in the UK, used the direct and indirect methods of testing and demonstrated the potential to increase their cut-off time of 2 minutes, allowing the inclusion of diluted semen stains [3,18,19]. These results show that it was possible to obtain a positive reaction for a 1 in 1000 dilution of semen as late as 10 minutes using the direct method, but for indirect testing, a dilution of 1 in 400 took 16 minutes to react [18]. There is evidence that the indirect method may give a positive reaction for the 1 in 40 dilution within a 2 minute cut-off [19]. Furthermore, research into different dispensing techniques of the direct method described a positive reaction for 1 in 3000 dilution within 29 seconds when testing cotton fabric [3].

Of note, this study reveals that both canine semen and certain food extracts reacted positively within the 50 s cut-off for human semen, which also fell within the 65 s cut-off for semen. In South African court, it was stated that a stain reacting positively within 50 s was considered to be from human and not animal origin. However, this specific use of the cut-off time remains unsubstantiated in the literature. Importantly, this study demonstrates that using a cut-off time for the brentamine test to discern between human and other specimens is misleading and is unlikely to yield accurate results.

The activity of AP for dogs has been reported as 1820.3 U/L or 1.820 U/mL [106], which is lower than the reported activity of human AP (250 – 1300 U/mL) [77,78,80]. Based on the activity of AP from other animals [80–83], it may be inferred that if semen from those species were tested using the brentamine FB method, the reaction times would fall within the cut-off time of 65 s, and the arbitrarily defined 50 s ‘human-specific threshold’, providing further evidence suggesting that the 50 second ‘human-specific’ cut-off time is inaccurate.

Furthermore, and surprisingly, the tested food extracts also reacted within 50 s. However, a positive presumptive test changes to purple and the colour change for most of samples, except mushrooms, was not the same colour defined as the presumptive positive. Therefore these samples would likely be excluded from further testing. These results are consistent with other investigations examining the specificity of the brentamine test, which showed that food extracts also reacted within their cut-off times, but the colours produced were inconsistent with a presumptive positive for semen [3].

Here, we show that the use of spectrophotometry is an objective method to determine the presence of AP in a sample, by using the rate of the reaction as a marker. The rates of the human semen dilutions were relatively higher than those of non-human samples except for Ceylon tea. Using the UV-Vis spectrophotometer to monitor the reaction at a specific wavelength of light is more accurate than by simple visual observation [85]. UV-Vis spectrophotometry has been used as a method to determine the presence of blood [84], and more recently a method has been produced to discern the presence of saliva in stains [85] but this method must still be validated for forensic use. One caveat of this method is that mushroom extract reacts with brentamine at the same peak wavelength as semen. A requirement for a UV-Vis spectrophotometer is that the path of the emitted light must pass through a liquid solution in the cuvette without passing through any air. This means there must be a minimum volume in the cuvette, and in this study the minimum volume was 1 mL (800 μ L of sample and 200 μ L of AP reagent). In the forensic context, 800 μ L would be considered a large volume, as often less sample is obtained, and it would also need to undergo other downstream analysis; e.g. forensic DNA profiling. Mini- and micro-spectrophotometers are available which require smaller sample volumes but to our knowledge, they are not designed to measure the change in absorbance over time [107–109]. Microspectrophotometers are designed to measure the spectra of microscopic objects or microscopic areas on specific objects [110]. These spectrophotometers are attached to microscopes, and the spectra is measured from light transmitted through the objective of the microscope from slides on the stage [111,112]. A portable handheld spectrometer has been designed but it measures Raman spectra not absorbance spectra and shows potential for body fluid identification at the crime scene [2,113]. Therefore the UV-Vis spectrophotometer used was used in this study as it has the ability to measure the change in absorbance over time which is what was required.

The scoring of slides of semen stains indicated no visible differences between fresh and aged specimens. In the aged specimens, spermatozoa with tails attached were seen until the 1 in 40 dilution factor. The presence of attached tails has been proposed to indicate a time frame for that stain, but this method was developed for the indication of time since intercourse [14,22,23]. It has also been suggested that the method for extraction of the spermatozoa could lead to the mechanical degradation of the tails [14]. This suggests that using tails as a marker of time might not be suitable for forensic investigations [14,24].

We were able to demonstrate that staining with haematoxylin and eosin can be used to determine the morphology of spermatozoa, which could aid in the identification of the species of mammal based on the stain. Using morphometric assessment, the dimensions of the spermatozoa heads of canine were significantly different to the heads of human spermatozoa. The calculated dimensions, however for canine and human fell outside of the literature values [40,114], but this is more likely due to the small sample size measured in this study ($n = 4$). Morphometric assessment has been shown to work as a computer assisted program to distinguish between abnormal sperm heads in both canine and human semen [36,114].

There were several limitations in this study. There was a small sample of canine semen specimens ($n = 2$), this was due to the lack of availability of these samples within the time frame in which the study was performed. The extinction co-efficient of the azo dye was unknown and this constant would be needed to convert the absorbance value to a concentration. This would have made the reaction kinetics more reliable as a marker for presumptive testing given that absorbance is an arbitrary unit, whereas concentration gives a more quantitative result.

We found that the direct method of presumptive semen identification is more sensitive than the indirect method. We show there is a chance for non-human semen and non-semen sample to react within the current cut-off time for presumptive identification of human semen. We illustrate the use of the UV-vis spectrophotometry to be a more objective apparatus in determining between human semen and canine semen as well as non-semen samples. This study also shows that it is possible to differentiate between spermatozoa from different mammalian species when doing forensic sperm cytology.

5 Conclusions

In this study, we show that direct testing is the more sensitive method for the presumptive presence of semen. Furthermore, sperm cytology could be used to distinguish between species but further investigations are needed to validate this method within a South African context. Importantly, we demonstrate that the cut-off time for supposed 'human specificity' is unsuitable, as human semen, canine semen and food extracts all react with the 65 s and 50 s thresholds. Furthermore, we propose using a UV-vis spectrophotometer as a more objective and accurate method to determine the presence of semen, and enzyme kinetics could be exploited to potentially differentiate between human and animal samples.

6 Supplementary Figures and Tables

Table S1: Mean reaction times with standard deviation (s) for each specimens when tested using the brentamine reagent

			neat	1/4	1/10	1/40	1/100	1/1000	1/2000	1/4000
Human (n=15)	Direct	Fresh	2.00±0.98	5.17±1.95	8.75±2.49	17.50±7.20	32.09±13.32	175.89±117.65	105.00±42.52	240.00±120.70
		Aged	2.50±1.35	4.83±1.99	9.75±5.72	18.92±10.15	41.50±36.54	148.00±70.71	178.50±95.87	355.00±77.78
	indirect	Fresh	8.14±5.30	26.29±14.33	58.71±43.10	168.71±76.59	214.33±55.82			
		Aged	6.93±1.98	19.93±12.21	51.07±29.53	130.00±50.28	238.00±87.68			
Canine (n=2)	Direct	Fresh	18.50±2.12	110.00±42.43	133.50±23.33	210.00	0 ^b			
		Aged	69.00±38.18	140.00	0 ^a	279.50±113.84				
	indirect	Fresh	36.00±8.49	190.00	0 ^b	360.00	0 ^b			
		Aged	240.00±42.43							

Purified AP		600 U/mL	300 U/mL	100 U/mL	50 U/mL	20 U/mL	10 U/mL	5 U/mL
	Direct	5	12	19	26	67	134	177

		Neat	1/2
Food Extracts (n=3)	Mushroom	33.00±8.89	37.67±9.29
	Rooibos	40.67±4.51	111.67±41.93
	Ceylon	2.00	0 ^a
	Red Grape	5.00±3.61	9.00±9.54
	White Grape	8.00±3.61	28.33±7.64
	Apple	9.00±3.00	16.67±2.52
	Ceylon	40.33±10.50	73.00
	Mushroom	48.33±16.07	63.33±20.82

^a there is no standard deviation due to the readings being the same value

^b there is on standard deviation as there was only one reading for that condition

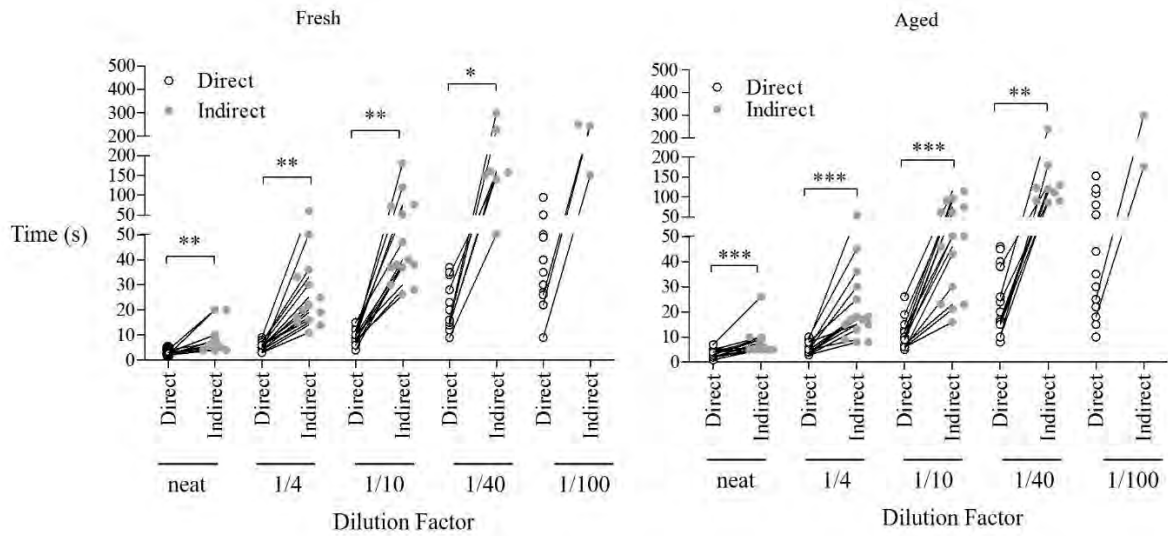


Figure S1: The human paired reaction times for the fresh and aged specimens for the direct (white) and indirect (grey) testing. Wilcoxon match testing was performed for each dilution comparing direct and indirect testing, significant differences are illustrated by the asterisk. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$

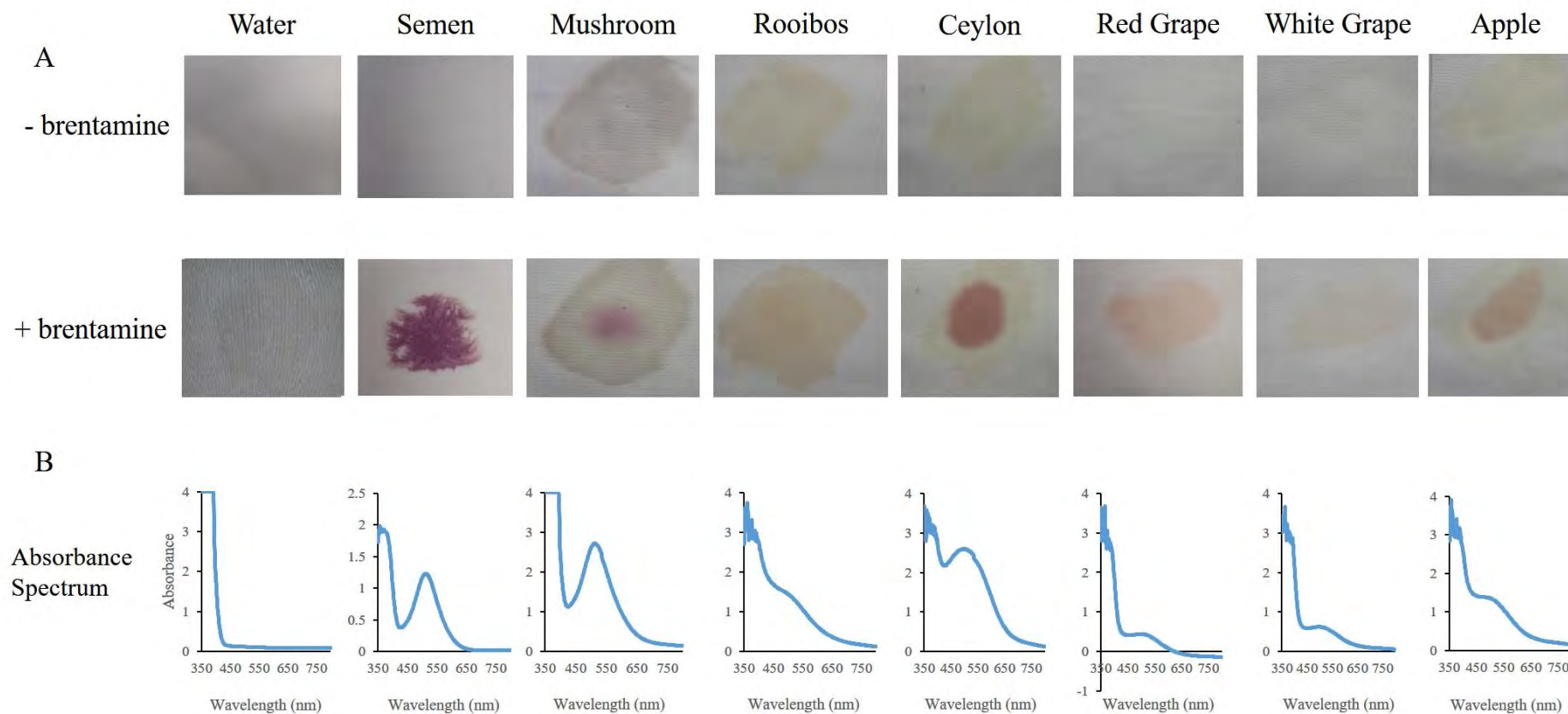


Figure S2: A) The colour change of the food extracts after direct treatment with the brentamine reagent B) Absorbance spectra of the colour reaction for each of the food extracts. Water acted as the negative control and semen was the positive control

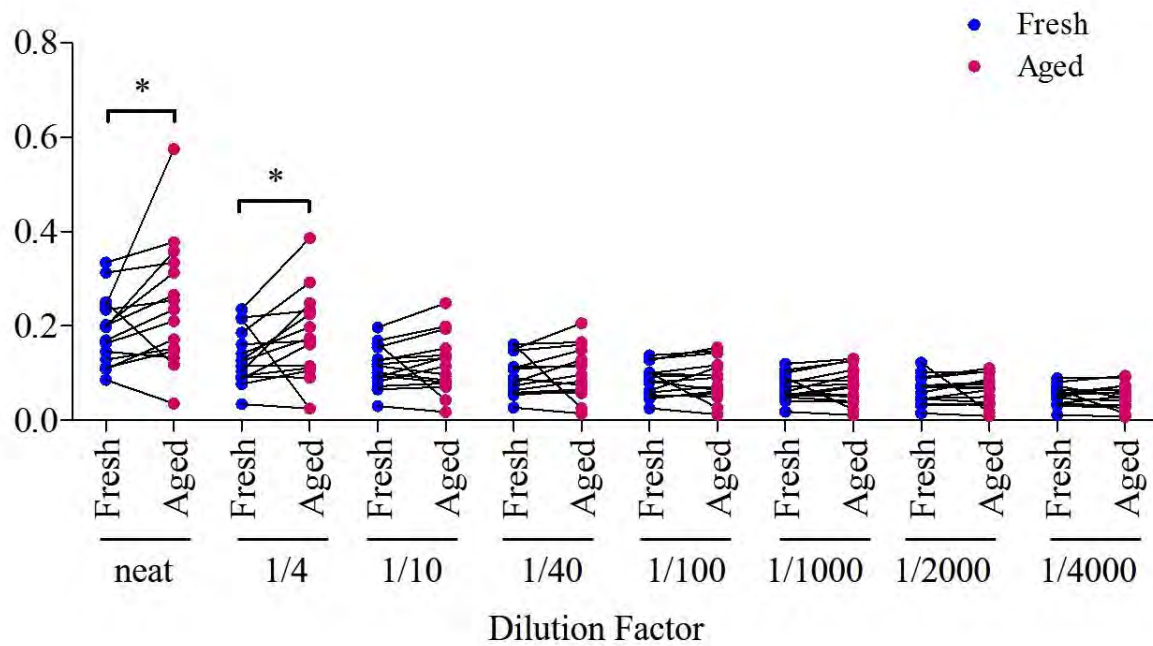


Figure S3 Rates for each reaction for paired fresh (blue) and aged (red) specimens for each dilution factor. (* $p < 0.05$). There is a statistical significant difference between fresh and aged reaction time for the neat and 1 in 4 dilution factors when tested using a Wilcoxon paired test.

Table S2: The mean reaction rate for each specimen with the standard deviation (Abs/s)

		1/100	1/200	1/300	1/400	1/500	1/600	1/800	1/1000
Human	Fresh (n=14)	0.1926±0.0733	0.1343±0.0581	0.1140±0.0443	0.0970±0.0432	0.0831±0.0336	0.0688±0.0275	0.0646±0.0291	0.0520±0.0210
	Aged (n=15)	0.2454±0.1337	0.1705±0.0980	0.1191±0.0620	0.1015±0.0540	0.0865±0.0431	0.0738±0.0380	0.0628±0.0318	0.0512±0.0259
Canine	Fresh (n=2)	0.0162±0.0057	0.0089±0.0040	0.0052±0.0021	0.0041±0.0011	0.0032±0.0010	0.0021±0.0008	0.0016±0.0008	0.0018 0 ^a
	Aged (n=2)	0.0144±0.0039	0.0078±0.0025	0.0050±0.0016	0.0035±0.0004	0.0031±0.0011	0.0021±0.0008	0.0017±0.0005	0.0013±0.0004
		10 U/mL	5 U/mL	2.5 U/mL	1 U/mL	0.5 U/mL	0.25 U/mL	0.125 U/mL	0.0625 U/mL
Purified AP		0.2288	0.1067	0.097	0.0645	0.0232	0.0115	0.0049	0.0027
Food extracts		1/10	1/20	1/50	1/80				
Mushroom		0.0351	0.0313	0.0233	0.0186				
Rooibos		0.0027	0.0008	0.0003	0.0004				
Ceylon		0.1807	0.1073	0.0306	0.0225				
Red Grape		0.0036	0.0014	0.0003	0.0004				
White Grape		0.0007	0.0006	0.0003	0.0002				
Apple		0.0187	0.0091	0.0011	0.0008				

Table S3: The Morphometrics of sperm heads for human (n = 4) and canine (n = 4)

	Human		Canine	
	Length	Width	Length	Width
	5.68	4.21	7.95	5.43
	4.68	3.62	7.29	4.91
	5.43	3.60	7.85	4.73
	5.31	4.63	7.62	5.23
Mean	5.28±0.43	4.02±0.50	7.68±0.29	5.08±0.31

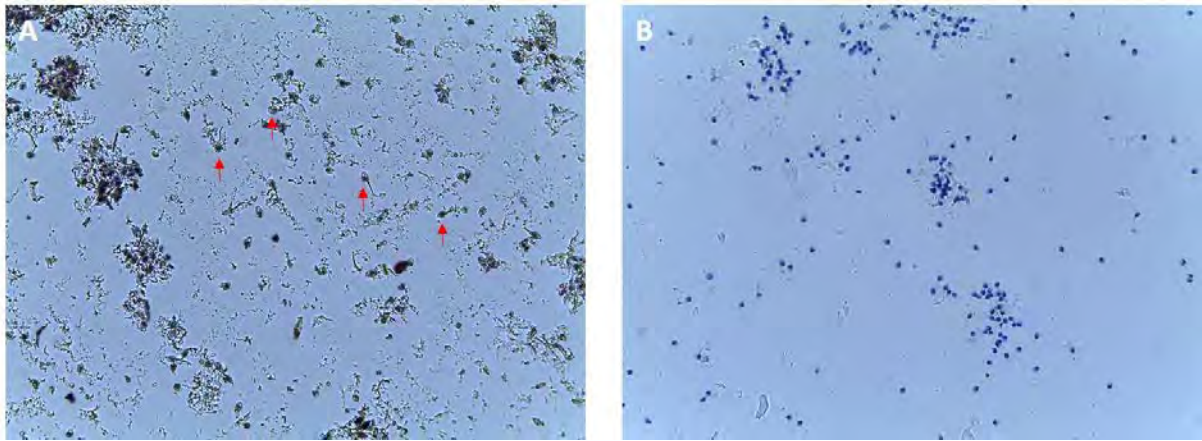


Figure S4: Spermatozoa extracted from A) fabric directly tested with brentamine FB (red arrows indicate spermatozoa) and B) untested fabric

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- [2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

- [3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z.Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

- [4] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2003 (accessed 13.03.03).

Part D. Appendices

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Budget and Ethics Approval Letter

Item	Quantity	Price (Rand) excl VAT
α -naphthyl phosphate	5g	1337.90
Brentamine FB	100g	5723.03
Fabric	3m	117.00
Spray bottles	6	76.00
Whatman Filter Paper	10 boxed	1094.40
Plastic Consumables (e.g. tips)	various	500.00
Acid phosphatase enzyme	1mg	10 807.20
Total (including VAT):		22 407.30



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee



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27 October 2015

HREC REF: 502/2015

Ms L Heathfield
CLS, Forensic Medicine
Level 1, Entrance 3
Falmouth Building

Dear Ms Heathfield

PROJECT TITLE: THE EVALUATION OF TESTS FOR THE IDENTIFICATION OF SEMEN-(MPhil candidate-L Curry)

Thank you for your response letter to the Faculty of Health Sciences Human Research Ethics Committee dated 15 October 2015.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th October 2016.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student L Curry will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH

2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki guidelines.

The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.